THE ROLE OF OLIGOSACCHARIDES IN COGNITIVE DEVELOPMENT

BY

STEPHEN ANDREW FLEMING

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Neuroscience
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2019

Urbana, Illinois

Doctoral Committee:

Associate Professor Ryan N. Dilger, Chair
Assistant Professor Naiman A Khan, Co-Chair
Professor Sharon M. Donovan
Research Assistant Professor Carolyn J. Butts-Wilmsmeyer
Dr. Brian M. Berg

Abstract

Not all mothers are able to breastfeed and there is significant evidence demonstrating breastfed infants outperform their formula-fed peers in cognitive development. Beyond the disparity between these two populations, human milk is one of the most complex foods, acting as the sole source of nutrition for infants and containing thousands of bioactive components. One of these components, the third highest by dry matter content, is oligosaccharides. Humans milk contains the most concentrated and diverse number of oligosaccharides than any other mammal known. They function to enhance immunity, protect against pathogens, and potentially stimulate brain development. The mechanisms by which these indigestible and fermentable carbohydrates impact the brain is largely unknown, however. Reports from rodent research suggest oligosaccharides in milk can improve memory and protect against anxiety. To investigate these possible effects, we used the pig as an animal model. The pig is a large animal with strikingly similar neuroanatomy and gastrointestinal physiology to the human. We demonstrate that the pig can be used to investigate the development of recognition memory and use this to explore the impact of various oligosaccharides on cognitive development. Ultimately, we demonstrate a relationship between fermentative metabolites of oligosaccharides, volatile fatty acids, and recognition memory. This relationship may be one of many potential axes within the gut-brain-axis. The results from this body of work should inform future research in the exploration of the gut-brain-axis and may point towards potentials modes of entry for modulation of this system.

Acknowledgements

It is only through an elaborate connection of dispassionate steps including discomfort, social pressure, and happenstance that brought me to completing my PhD. I wish I could state that I have made it to this point through ambition, vision, and hard work, however I can't shake the feeling that I have simply interacted with the inputs of my life and have provided this document as a substantial output. Regardless, this thesis fills me with pride. I am forever indebted to my wife, Rachel Fleming, for believing in me and persuading me to do more with my life than meet the minimum requirements. I had no direction after high school and partway through community college started "Fleming's Lawn Mowing Service"" to pay for school. After a year of toil outside I realized building my own lawn mowing empire was perhaps not the future I wanted. Thus, after discomfort and significant sunburn in that position I followed my wife to the University of Illinois where I became intrigued by neuroscience. There I met the next crucial player, Amogh Belagodu, who I spent long hours in the lab with performing monotonous scientific tasks as an undergraduate.

A few years of great company and routine after-lab-beers were enough to convince me that the intellectual pursuit of science could outweigh the sheer amount of dishwashing and pipetting that science often requires. Despite again having little direction, both Amogh and Rachel pressured me toward applying for Graduate School. I ended up staying in Urbana-Champaign after meeting my now advisor and great mentor, Dr. Ryan Dilger. Ryan was the last in a long list of professors I interviewed with, and I only happened upon his website after striking out from almost every other interview. He has a peculiar ability to gloss over the fact pigs smell and that to study nutrition means to study excrement. Such skills came in handy for convincing a young, fresh, Chicago suburb graduate to work on a research farm. This position has led me to lifelong friends, international travel, expanded my confidence, broadened my perspectives, and allowed me to build a host of skills that I hope are evident in this thesis. Thus I am greatly indebted and grateful for Ryan's mentorship these past five years.

To think had I made more money mowing lawns I might have said no to the events preceding this. I must of course thank my family, my parents Mark and Felicia and brothers Josh and Matt. I had an incredible childhood and am fortunate to be blessed with such a fun-loving and supporting family. Despite having to spend a couple of years convincing my family that watching hundreds of hours of pigs sniffing objects was "research" they believed in me anyways. I also want to thank my in-laws, Ken and Michelle Freedman. I am truly lucky to have not one but two sets of loving parents and families. To my labmates, thank you for indulging me in a seemingly endless list of "would you rather?" questions when we should have been working and being there for me when needed. I of course must thank my committee. Thank you to Brian Berg and Sharon Donovan, who have provided in no small amount mentorship and guidance throughout my graduate career. I have you two to thank for the abundance of industry exposure that many graduate students don't get to take advantage of. Thank you to Naiman Khan and Carrie Butts-Wilmsmeyer for entertaining my scientific questions. To all the others who have supported me along the way, thank you. Lastly, I thank my

wife Rachel for having the endurance to be married to me, her goofy personality, her unwavering support, and her infectious laugh.

Contents

Chapter 1

Introduction

1.1 A Primer on Early-Life Nutrition and Brain Development

It is widely accepted that human milk is the preferred source of nutrition for infants, however infants that are unable to receive human milk must rely on infant formula as either a partial or whole source of nutrition (Martin et al., 2016). Human milk has been shown to have numerous benefits in comparison to infant formula in stimulating the growth and development of gastrointestinal and immune systems (Ballard and Morrow, 2013). Multiple reports from cohort studies investigating cognitive development is altered between breastfed and formula fed babies have shown a positive benefit of breastfeeding. After controlling for sociodemographics, maternal intelligence, and home environment, longer breastfeeding duration has been associated with higher Peabody Picture Vocabulary Test scores at 3 years of age and improved performance on the Kaufman Brief Intelligence test at 7 years of age (Belfort et al., 2013). This effect was specific to these intelligence tests as breastfeeding duration was not associated with the Wide Range Assessment of Memory and Learning Scores.

In a systematic review of 17 studies it was found that breastfeeding is related to improved performance on intelligence tests (Horta et al., 2015). Of the 17 studies the majority were cohort study designs, and a range of standardized cognitive tasks were used (e.g., the Bayley Scales of Infant Development, Wechsler Full Scale, Peabody Picture Vocabulary Test, and more). Breastfed babies demonstrated higher IQ, but this effect was reduced (yet still significant) when maternal IQ was included in the model. Furthermore, this relationship was weaker in 10-19 year old subjects than in 1-9 year old subjects. Results from neuromaging studies support these findings, as infants exclusively breastfed for 90 days demonstrated greater mylenation in several brain regions than their formula fed counterparts (Deoni et al., 2018). Still, a study using propensity score matching demonstrated no association between cognitive outcomes at 3 and 5 years of age when infants were fully breastfed up to 181 days. Infants fully breastfed for greater than 181 days demonstrated reduced

hyperactivity at 3 but not 5 years of age. At best, this study showed that what few benefits that do occur are transient (Girard et al., 2017). Despite some conflicting evidence on when and to what extent breastfeeding influences cognitive development, the evidence that support a positive effect of breastfeeding appear to outweigh those suggesting neutral effects.

The mechanisms by which breastfeeding improves cognition are myriad, with much to discover regarding the relationship between intake, metabolism, and cognition. The infant brain accretes and utilizes several compounds in greater concentrations than adults, and these include gangliosides, long-chain polyunsaturated fatty acids (LCPUFA), and choline. Gangliosides are highly concentrated in brain matter and constitute 10-12% of the lipid matter in the neuronal membrane, and accretion in the developing brain begins during the 10^{th} week of gestation up to 5 years of age (Svennerholm et al., 1989). Gangliosides play a critical role in functions such as synaptic transmission, plasticity, neurogenesis, synaptogenesis, cell proliferation and cell differentiation (Palmano et al., 2015). The role of LCPUFA in infant development has also been of great interest, especially their relation to cognition. LCPUFA accretion is greatly increased in the third trimester and LCPUFA are rapidly incorporated into gray matter throughout the first 2 years of life (Hoffman et al., 2009). This has led to significant research on the relationship between LCPUFA and early life cognition. Lastly, there is significant evidence that choline plays an important role in early life. Choline status during pregnancy and lactation has been shown to impact memory, cell division, apoptosis, and differentiation (Zeisel, 2004).

While there is mounting evidence that components of human milk including DHA (Hoffman et al., 2009), choline (Zeisel, 2004), and gangliosides (Palmano et al., 2015) support brain development, emerging research suggests milk oligosaccharides (ranging from 3–32 monosaccharide units in length (Morrow et al., 2005)) may contribute to brain development as well (Bode, 2012). As the third most abundant component of human milk, oligosaccharides (OS) are thought to aid in gastrointestinal development, brain development and prevention of pathogenic events (Bode, 2012). Known differences in OS concentration and composition exist between human milk and milk from other mammalian species. Notably, mature human milk contains between 3.5–14 g OS/L, whereas mature bovine milk, which is often used as a base for infant formula, contains only 0.3–0.5 g OS/L and infant formulas are reported to contain 0.4–8.0 g OS/L (Ten Bruggencate et al., 2014). Additionally, 50–70% of human milk OS are fucosylated (i.e., contain a fucose molecule), followed by 10–30% sialylated human milk OS (i.e., contain a sialic acid (SA) molecule) and approximately 10% of human milk OS are neutral OS that do not contain either fucose or SA (Ninonuevo et al., 2006). Some of these oligosaccharides feed gut bacteria and act as prebiotics, and evidence suggests that the incorporation of prebiotic substrates into infant formula may have beneficial effects on intestinal function (Bertelsen et al., 2016, Vandenplas et al. (2013)). In fact, the function of prebiotics appears to go beyond aiding the development of the gastrointestinal tract and has profound effects on neurobiology and cognition in both animal and human models (Kao et al., 2016, Sampson and Mazmanian (2015)). The understanding

of the mechanisms is largely unknown, yet several hypotheses have been put forth (Martin et al., 2018, Sampson and Mazmanian (2015), O'Mahony et al. (2015), Lyte (2013)). Here we review the evidence for prebiotic mediated changes in neurobiology, mechanisms of action within the gut-brain-axis, and how the pig as a biomedical model may be used to investigate the relationship between early-life nutrition and cognitive development.

1.2 Evaluation of the impact of various prebiotics on cognition and brain development

1.2.1 Introduction

Prebiotics are ingredients that resist gastric and intestinal digestion and absorption by mammalian enzymes, are fermentable by host microflora, and selectively stimulate the growth and activity of intestinal bacteria associated with health and well-being (Gibson et al., 2010). Currently, the most common prebiotics added to infant formulas are galactooligosaccharides (GOS), fructooligosaccharides (FOS), and polydextrose (PDX) (Bertelsen et al., 2016). However, recently human milk oligosaccharides have been targeted as promising components for addition to infant formula (Vandenplas et al., 2018). There is substantial evidence suggesting that each of these prebiotics alone or in combination stimulate the microbiota and bring development of the GI track of formula fed infants closer to that of breastfed infants. Furthermore, intake of these prebiotics has also been shown to positively impact cognitive and affective behaviors (Sampson and Mazmanian, 2015). Currently, most prebiotic research related to cognition has been conducted on GOS, FOS, and human milk oligosaccharides (HMO) (Kao et al., 2016).

1.2.2 Evidence for GOS and FOS

1.2.2.1 Alteration of molecular phenotypes by GOS and FOS

Galactooligosaccharides are defined by their galactose backbone and degree of polymerization (DP) up to 9 (Van Leeuwen et al., 2016) and can be found in various linear or branched combinations. Fructooligosaccharides are breakdown products of their longer chain fiber inulin (Kao et al., 2016) and are fructose polymers that exist in a DP up to 10. Both FOS and GOS are indigestible and selectively fermented in the colon. There is relatively more research on the cognitive benefits of GOS than there is for FOS, however both have been studied. Recent evidence for GOS and FOS has repeatedly established a role for both prebiotics to alter the molecular expression of learning related proteins. Oral gavage with 3 or 4 g/kg FOS or GOS for 5 weeks has been shown to differentially alter central brain derived neurotrophic factor (BDNF), N-methyl-D-aspartate receptor (NMDAR), and plasma D-serine in adult male rats (Savignac et al., 2013). BDNF and NMDAR NR1 were greater in the hippocampus of those fed FOS, whereas NR1 was greater in the frontal cortex and NR2 greater in the hippocampus of those fed GOS. The authors

attributed some of these changes to an elevation in plasma D-serine, a co-agonist of glutamatergic receptors. Importantly, the authors additionally quantified the bifidogenic effect of GOS and FOS, demonstrating that GOS increased *bifidobacteria* counts 80% above controls, whereas FOS increased *bifidobacteria* 25% above controls. Unfortunately, the dosage of each was not controlled for, as GOS was fed at 4 g/kg whereas FOS was fed at 3 g/kg.

The bifidogenic effect of GOS and FOS is insufficient to explain the results on BDNF and NDMAR. Even though *bifidobacteria* counts correlated with NR1 subunit expression, FOS had a lower bifidogenic effect while still stimulating expression of BDNF and NR1 in the hippocampus while GOS did not. It may be obvious but worth stating however that *bifidobacteria* is one of many strains of bacteria that may have been altered. An interaction with the anorectic hormone Peptide YY (PYY) may partially explain the effect of GOS on BDNF. While FOS was shown to increase BDNF protein in the hippocampus, treatment with GOS increased BDNF mRNA in the dentate gyrus, while decreasing BDNF mRNA in the CA3 region of the hippocampus. Only treatment with GOS increased circulating PYY, and when cultured SH-SY5Y cells were treated with plasma from groups given water, FOS, or GOS, only plasma from animals provided GOS increased BDNF expression above controls. Plasma from animals treated with FOS also increased BDNF, but this effect did not reach statistical significance. Together, this provides evidence that the prebiotics GOS and FOS may be acting through a hormonal route to alter BDNF expression in the brain. Furthermore, this study provides evidence that both GOS and FOS can induce molecular changes in the adult brain, however the lack of equal dosage confounds the ability to attribute these effects to their general prebiotic activity, or specificity of the prebiotics themselves.

In a follow up study, the same dose of GOS (4 g/kg) provided by oral gavage during neonatal development (postnatal days (PND) 3-21) increased BDNF, synaptophysin, and the NMDAR subunit GluN2A at PND22, which persisted after supplementation ended up to PND56 (Williams et al., 2016). Interestingly, the authors noted that other structural proteins of interest such as microtubule-associated-protein-2 (MAP2) and growth-associated-protein-43 (GAP43), proteins related to growth and maturity of the synapse, were unaffected by diet. The authors interpreted such results as an indication that feeding GOS does not alter structural components of the brain, but rather their molecular components.

To investigate potential mechanisms of B-GOS mediated changes in protein expression, the same group compared B-GOS and acetate (both at 0.5 g/kg/d in drinking water) supplementation in female adult Sprague Dawley rats and found that B-GOS inhibited combined cortical and hippocampal homogenate histone deacetylase (HDAC) activity while paradoxically increasing cortical mRNA of HDAC-1 and HDAC-3. Supplementation with sodium acetate inhibited histone acetyltransferase (HAT) activity in a combined cortical and hippocampal homogenate, had no effect on HDAC1-4 activity, increased HDAC3 and HDAC4 specifically in the hippocampus, but had no effect on NMDAR subunits or 14 genera of fecal bacteria. While dietary supplementation with both B-GOS and acetate increased plasma acetate, the authors concluded

that the effects of BGOS on HDAC and NMDAR expression may be independent of microbial production of acetate (Kao et al., 2019).

1.2.2.2 Impact of GOS and FOS on cognition

GOS supplementation in drinking water was later shown by the same group to reduce lipopolysaccharide (LPS) induced anxiety and normalize serotonin receptor and cytokine expression (Savignac et al., 2016). After administration of LPS, those fed GOS had similar latency to enter the light area of a light/dark box to saline controls and spent a similar amount of time in the light compartment as saline controls, suggesting GOS supplementation brought anxiety closer non-LPS induced levels. Additionally, animals fed GOS had reduced expression of the cytokines IL-1 and IL-6 and serotonin receptor 5-HT2AR in the frontal cortex after LPS stimulation. While previous studies used an oral gavage of 4 g/kg GOS, this study included GOS (via BGOS, a Bimuno® product containing 43% GOS) in drinking water at rate of approximately 13g/L, accounting for purity of the GOS product the final concentration was approximately 6.25g/L. It is difficult to compare these doses as a final g/kg BW calculation was not conducted. In both studies by Savignac et al., (Savignac et al., 2013, Savignac et al. (2016)) *bifidobacteria* counts were obtained, with the oral gavage of 4 g/kg increasing counts by an additional 80% of control levels, whereas included in drinking water GOS increases *bifidobacteria* by an additional 200% of control levels, suggesting the dosage provided in drinking water may have had a stronger bifidogenic effect. The authors suggest that *bifidobacteria* may not express microbe-associated molecular patterns that fully activate host enteric toll-like receptors, and the proliferation of *bifidobacteria* may have played a large role in reducing inflammation. Finally, the authors note that past work has shown that LPS induces elevations in 5-HT2AR in rat cortex. The GOS mediated reduction in 5-HT2AR may attribute to reduced expression of behavioral anxiety, however evidence for a strong mechanistic action is lacking. Overall, GOS supplementation reduced LPS induced increases in anxiety, cytokine expression, and serotonin receptor upregulation.

A later study further investigated the impact of dietary GOS on cognition and found GOS (fed 3% GOS in drinking water) improved attentional set shifting performance in adult male Sprague Dawley rats (Gronier et al., 2018). Furthermore, *in vivo* iontophoresis in the prefrontal cortex demonstrated increased NMDA response in the GOS fed group, and those fed GOS demonstrated resistance to NMDA antagonism. While B-GOS increased cortical GluN2b, plasma acetate, and cortical acetyl CoA carboxylase, there was no effect on cortical HDAC expression, contrary to the same group's findings in female rats (Kao et al., 2019).

Strong evidence for GOS on cognition may come from a human study in adults. Male and female participants were instructed to take 5.5 g/day of GOS, FOS, or maltodextrin each day for 3 weeks. After supplementation participants were tested on a battery of cognitive tests to assess emotional processing and salivary cortisol was collected upon waking to quantify the salivary cortisol waking response. Galactooligosaccharide supplementation reduced the waking cortisol response and reduced attentional vigilance towards negative

stimuli as compared to maltodextrin or FOS supplemented controls. Mechanisms for these effects are proposed to be related to gut-brain-axis regulation of the HPA axis (Dinan and Cryan, 2012) or possibly are vagal related (Bravo et al., 2011), however evidence for such theories are lacking. Nevertheless, this study provides stronger evidence for the effect of GOS on cognition than the previous reviewed as it was conducted in a human model and controlled for dosages of FOS and GOS. While in a human model and using different methods, this study builds upon the previous study demonstrating that GOS has anxiolytic effects, here from the reduction in waking cortisol and reduction in attention towards negative stimuli.

While FOS has been shown to affect the brain in similar ways as GOS, its effect on behavior is less substantial. However, recently it was demonstrated in a mouse model of Alzheimer's Disease (AD) that FOS supplementation increased performance in the Morris water maze, suggesting FOS may exert anti-aging effects (Yen et al., 2017). These effects were similar to another study that showed that chitosan oligosaccharides (COS) reduce the cognitive deficits in a rat model of AD (Jia et al., 2016). While it has been shown that FOS does not affect anxiolytic behavior but does improve learning and memory, GOS appears to improve both learning and memory (Gronier et al., 2018) in addition to its anxiolytic activity (Savignac et al., 2016).

1.2.3 Impact of prebiotic blends on neurodevelopment

While GOS alone exerts several effects on molecular expression of learning related proteins in the brain and anxiety-related behaviors, GOS has been shown to work in combination with polydextrose (PDX) and milk bioactives to alter brain development. Supplementation with a combination of GOS, PDX, milk-fat globule membrane (MFGM), and lactoferrin in neonatal piglets for 29 days was shown to alter structural brain development while minimally influencing behavior (Mudd et al., 2016a). Piglets fed this combination had altered gray and white matter concentrations in several brain regions, with control pigs exhibiting greater grey and white matter in several clusters of the cortex, possibly due to an alteration of the synaptic pruning process. Additionally, piglets provided the test article had reduced radial and mean diffusivity in the internal capsule, a measure of greater maturation. Although GOS has been shown to increase concentrations of central BDNF in several studies, BDNF mRNA was not upregulated in either the right hippocampus or right frontal cortex. Furthermore, the authors observed no differences in learning and memory on the spatial T-maze.

In a study examining the same ingredients as the previous study, Waworuntu et al., (Waworuntu et al., 2016) demonstrated that weanling rats provided prebiotics and MFGM for 40 days displayed alterations in neuronal spine morphology in the dentate gyrus of the hippocampus and medial prefrontal cortex. Supplemented rodents had increased spine length and head diameter in apical dendrites 200 µm from the soma in the medial prefrontal cortex, whereas there was a proliferation of thin and mushroom type spines in the dentate gyrus. The results are difficult to interpret, as alterations in morphology have been associated with both

deficits and advancements in cognitive function. Interestingly, this study in combination with Mudd et al., (Mudd et al., 2016a) contrast with previous work on GOS. Williams et al., (Williams et al., 2016) noted that GOS did not alter proteins related to structure and suggested that GOS may be acting to alter the molecular phenotype but not structural development, whereas Mudd et al., (Mudd et al., 2016a) and Waworuntu et. al., (Waworuntu et al., 2016) together show structural alterations but not molecular or functional alterations in the brain.

1.2.4 Evidence for Human Milk Oligosaccharides

The two HMO most well studied in their relation to cognitive function are sialyllactose (SL, 3' and 6') and 2'Fucosyllactose (2'FL). SL is an oligosaccharide composed of lactose bound to sialic acid in the either the 3 or 6 carbon position. 2'FL is an oligosaccharide composed of a glucose and galactose backbone with fucose bound to galactose. Both fucose and sialic acid are present in glycoproteins found in the synapse (Wang, 2012; Zatz and Barondes, 1971), suggesting possible direct effects of integrating these residues in the brain.

1.2.4.1 Sialyllactose

A recent study demonstrated that dietary intake of SL increased sialic acid in discrete regions of the brain. Experimenters supplemented formula fed piglets with 2 g or 4 g of 3' or 6' SL for 21 days and found that total sialic acid was enriched in the corpus callosum when fed 2 g 3'SL compared to diets containing no SL. Interestingly, protein bound sialic was enriched in the hippocampus compared to a group fed a combination of PDX and GOS, but not controls. Overall the results demonstrated that feeding SL results in a dose, structure, and region-specific increase in brain sialic acid, however whether this has functional consequence for behavior remains to be seen. In a young adult mouse model investigating possible anxiolytic effects of SL, both forms of SL were found to reduce anxiety-related measures and bring performance back to control levels when mice were introduced to a social stressor. Mice provided SL and exposed to a social stressor exhibited similar exploration of the light component of the light/dark box as mice not exposed to a social stressor. Additionally, SL supplemented mice explored the open area of an arena for similar amounts of time as non-stressed animals, an indication of less fear and anxiety and greater willingness to explore. Furthermore, SL prevented a social stressor induced reduction in immature neurons, regardless of isoform. These anxiolytic effects are similar to those observed when mice are supplemented with GOS, however there is little known of the mechanisms behind their anxiolytic effects

There is relatively sparse data on the impact of sialic acid containing HMO on cognition, however given that sialic acid is a major constituent of gangliosides and glycoproteins, some indirect evidence for the impact of sialic acid containing HMO via their sialic acid residue may be found in studies investigating non-prebiotic sialic acid containing ingredients. Sialic acid plays a major role in the post-translational glycosylation of proteins and is a structural component of gangliosides. Gangliosides are highly concentrated in brain matter

and constitute 10-12% of the lipid matter in the neuronal membrane where they play a critical role in functions such as synaptic transmission, plasticity, neurogenesis, synaptogenesis, cell proliferation and cell differentiation (Palmano et al., 2015).

Dietary gangliosides fed to children with cerebral palsy for 3 months were found to improve symptoms such as muscle tension, limb function, language ability, and intelligence (Xu and Zhu, 2005). Additionally, the levels of serum gangliosides were brought closer to a breastfed reference group (Gurnida et al., 2012). Due to the use of a developmental human model this study provides strong evidence that sialic acid contributes to not only cognition but also motor development. Young piglets supplemented with sialic acid via casein glycomacropeptide from postnatal days 3-35 had dose-dependent increases in performance in the radial arm maze, with those provided the most sialic acid completing a difficult version of the task with the fewest mistakes. All groups fed sialic acid had enriched protein bound, but not ganglioside bound, sialic acid in the frontal cortex. Additionally, those fed the highest amount of sialic acid had increased levels of ST8SIAIV, a polysialyltransferase important in sialic acid metabolism. Sialyltransferase activity in the frontal cortex correlated inversely with number of mistakes on the radial arm maze, piglets with lower sialyltransferase activity made more mistakes. Despite this correlation, there were no dietary effects on sialyltransferase activity, suggesting sialyltransferase activity is not the mechanism by which performance was improved.

In a later study, piglets supplemented with lactoferrin, a sialic acid rich glycoprotein, from postnatal day 3-38 were found to have increased performance on the same version of the radial maze. More piglets in the treatment group were able to complete both easy and difficult versions of the task, with the piglets provided lactoferrin making fewer mistakes on the difficult version (Chen et al., 2015). Gene microarray in hippocampal tissue revealed those provided lactoferrin had an upregulated BDNF neurotrophic signaling pathway, affecting genes related to organization of the cytoplasm, cytoskeleton, growth of neurites, and anxiety. The impact on genes related to anxiety suggested lactoferrin may decrease anxiety. This would be in alignment with the previously discussed results from Tarr et al., (Tarr et al., 2015), wherein mice provided SL demonstrated attenuated anxiety when introduced to a social stressor.

Taken together, few studies have evaluated the impact of SL on cognition and behavior, however there is evidence from several other studies that sialic acid containing ingredients positively impact behavior. Evidence from studies evaluating various non-prebiotic sialic acid containing components suggest the cognitive benefit likely arises from a direct incorporation of sialic acid into the brain or modulation of plasticity or growth-related pathways.

1.2.4.2 2'Fucosyllactose

Similar to sialyllactose, 2'fucosyllactose contains a monosaccharide residue that is present in glycoproteins in the synapse, fucose. Earlier work in chicks demonstrated that fucose is incorporated into glycoproteins after a passive avoidance task (Sukumar et al., 1980), and soon after a study corroborated this finding in

rats, demonstrating that impairment of fucosylation in the rat hippocampus also impaired retention during a discrimination task (Jork et al., 1986). While neither of these studies demonstrate that dietary HMO, or even fucose itself, has a positive impact on cognition, these early studies provide evidence that fucose may play a role in learning and memory.

Two recent studies evaluated the impact of fucose and 2'FL supplementation on learning and memory in rodents. In the first, adult mice and rats were supplemented with 0.312% or 0.625% 2'FL for 5 or 12 weeks respectively, providing 350 mg/kg BW per day (Vázquez et al., 2015). Both mice and rats fed 2'FL showed increased and longer lasting potentiation of Schaffer collateral neurons in the CA1 region of the hippocampus. Supplemented mice displayed increased performance on a place learning, working memory, and fixed-ratio lever pressing task in an operant box, suggesting that multiple cognitive domains were enhanced by 2'FL supplementation. Expression of the protein postsynaptic density 95 (PSD-95) in supplemented rats qualitatively appeared increased upon immunohistochemical analysis in the hippocampus, frontal cortex, and striatum which was confirmed by western blot in the hippocampus and frontal cortex. Additionally, calcium/calmodulin-dependent protein kinase II (CaMKII), a protein involved in long-term potentiation, was increased in the hippocampus, and BDNF was elevated in both the hippocampus and striatum (Vázquez et al., 2015).

In a follow up study dietary 2'FL was compared to dietary L-fucose, and subdiaphragmatic bilateral vagotomy was used to assess the requirement of the vagus nerve for 2'FL mediated increases in cognition (Vazquez et al., 2016). Rats were supplemented with 2'FL and L-fucose at the same dosage as the previous study. In alignment with the previous study, rats provided 2'FL demonstrated improved potentiation, however those provided L-fucose did not. Vagotomy abolished 2'FL mediated increases in long-term potentiation (LTP), however all groups (sham/vagotomy and control/2'FL) were still able to perform above criterion in a fixed-ratio lever pressing task. There was a non-significant decrease in days to reach criterion in 2'FL/sham animals compared to 2'FL/vagotomy animals. By the end of training, 2'FL/sham animals displayed greater lever presses than 2'FL/vagotomy or control/vagotomy animals. Overall, vagotomy did not inhibit the ability to perform the task for any group, however it did prevent a 2'FL mediated increase in performance. These two studies provide the clearest evidence for mechanisms behind prebiotic enhancement of cognition. 2'fucosyllactose has been shown repeatedly to stimulate long-term potentiation and may be doing so via a vagal-dependent mechanism. Whether these findings remain true for all prebiotics remains to be seen. The lack of potentiation in animals provided L-fucose suggests that the increase in LTP is due to a prebiotic effect, not a direct effect of L-fucose.

1.2.5 Conclusions

All prebiotics reviewed here have shown data suggesting enhancements to cognitive function. However currently the greatest evidence lies with GOS and 2'FL. Galactooligosaccharide has repeatedly shown

anxiolytic like actions across mouse and human models as well as increased expression of proteins related to learning and memory, whereas 2'FL has repeatedly shown an ability to increase long term potentiation and learning and memory in several operant paradigms in rodent models. While GOS is already included in many infant formulas, it shows the highest promise of success for a randomized placebo controlled double blind study investigating its impact on cognition.

1.3 Gut-brain-axis communication

1.3.1 Introduction

The composition of the gut microbiota is highly variable and sensitive to the environment, as mode of delivery, term, and early nutrition have large impacts on microbial composition (O'Mahony et al., 2015). The gut microbiome is generally considered immature until 3 years of age (Clarke et al., 2014). During that developmental window, colonization of the gut may set the stage for protection from or risk of disease in later life (Clarke et al., 2014). Modulation of the microbiome via prebiotics and/or probiotics have been shown to alter behavior, increasing learning and memory or acting as an anxiolytic. The discovery of these effects has prompted an interest in the bidirectional communication between the gut and the brain, or the gut-brain-axis (GBA). In this review, potential mechanisms of communication between the two will be discussed, including the role of short chain fatty acids (SCFA), the serotonergic system, and the impact of prebiotics and probiotics.

1.3.2 Short Chain Fatty Acids

Short chain fatty acids are primarily formed by fermentation in the colon of carbohydrates and proteins by enteric anaerobic microbes (Cummings and Macfarlane, 1991), but mostly from carbohydrates. Acetate, propionate, and butyrate are the major SCFA produced by fermentation, however they are accompanied by formate, valerate (although linear is sometimes grouped with branched chain fatty acids (BCFA)), and caproate. Isobutyrate, 2-methyl-butyrate, and isovalerate are produced as products of fermentation of branched chain amino acids (Macfarlane and Macfarlane, 2003). However, a variety of bacteria ferment many amino acids, including: Glu/Gln, Asn/Asp, Lys, Ser, Thr, Arg, Gly, His and BCAA. Protein and peptides are deaminated, decarboxylated, and metabolized to SCFA, BCFA, phenols and indoles, organic acids, gas, and biogenic amines. (Dai et al., 2011). Lactate, ethanol, and succinate are also produced by fermentation, but are usually consumed by other microbiota and thus do not accumulate in the colon to an appreciable extent (Macfarlane and Macfarlane, 2003).

SCFA production is greatest in the proximal large intestine, where SCFA are mainly produced from degradation of carbohydrates. Acetate, propionate, and butyrate make up 95% of SCFA, and are typically present in molar ratios of 60:20:20 (den Besten et al., 2013). Short chain fatty acids still dominate by

concentration, but the relative proportion of carbohydrate decreases in the distal colon and fermentation of protein and amino acids leads to an increase in the relative concentration of BCFA (Macfarlane and Macfarlane, 2003). Broadly speaking, the majority of intestinal microbes utilize the glycolytic pathway to break down carbohydrate, while many other gut bacteria use the pentose phosphate pathway. Both pathways ultimately lead to the synthesis of pyruvate, which can be metabolized to acetate, propionate, or butyrate (Macfarlane and Macfarlane, 2003). Bacteria either engage in fermentative metabolism and ATP formation or substrate level phosphorylation. Fermentation reactions must be self-balancing regarding the formation and consumption of redox agents, resulting in largeer amounts of acetate formed and higher ATP yield (Macfarlane and Macfarlane, 2003). Substrate level phosphorylation are energetically inefficient as compared to oxidative metabolism and yield less ATP. Anaerobes that produce more reduced products (e.g., hydrogen, lactate, succinate, butyrate, and ethanol) yield less ATP and acetate, and vice versa.

As it is difficult to measure the flux of SCFA production and absorption in the colon, most data on production is from in vitro studies, which suffer from lack of absorption and accumulation of products and changes to the microbial environment (den Besten et al., 2013). Despite these drawbacks, *in vitro* work has demonstrated specificity of fiber/oligosaccharide source in regard to the amount and ratios of SCFA produced (den Besten et al., 2013). Although it appears there is a linear correlation between fiber intake and SCFA concentration in the cecum (Levrat et al., 1991) the specificity to species, fiber, age, anatomy, and microbial population make prediction difficult (Millet et al., 2010). High protein diets tend to increase the concentration of BCFA in the colon, whereas non-digestible carbohydrates like starch increase bacterial production of SCFA (Dai et al., 2011) .

There is relatively little known about the broader functional roles of BCFA, however SCFA function as HDAC inhibitors, sources of energy, and modulators of metabolism in a variety of organs (den Besten et al., 2013, Bourassa et al. (2016), Koh et al. (2016), Macfarlane and Macfarlane (2011), Selkrig et al. (2014)). SCFA can be absorbed and act on distant organs via a humoral route (Selkrig et al., 2014) as well as directly on vagal afferent neurons (VAN) (Forsythe et al., 2014). The following sections will review the role of SCFA as histone deacetylase inhibitors and regulators of blood-brain-barrier integrity.

1.3.3 SCFA, HDAC inhibition, and the blood-brain-barrier

All SCFA act as HDAC inhibitors, with butyrate and propionate acting more strongly than acetate (Selkrig et al., 2014). HDAC inhibitors prevent transcriptional silencing by blocking the removal of acetylated residues from DNA which causes a tightening of chromatin structure and transcriptional silencing. In the presence of HDAC inhibitors global histone acetylation increases. Inhibition of acetylation is not restricted to histones, with over 1700 and 4000 proteins identified in humans and mice, respectively (Choudhary et al., 2009, Baeza et al. (2016)).

Many neurodegenerative diseases have been associated with lower histone acetylation (Bourassa et al.,

2016), therefore the use of HDAC inhibitors may be therapeutic. The vast majority of research examining the therapeutic potential of SCFA, namely butyrate, has been conducted by administering butyrate intraperitoneally (Stilling et al., 2016), thus bypassing the gastrointestinal tract. However, some have shown that butyrate in drinking water or by oral gavage (Minamiyama et al., 2004, Bonthuis et al. (2011), Erny et al. (2015)), and others have shown that supplementation with "butyrate producing" bacteria have also improved cognition (Liu et al., 2015, Sun et al. (2016a), Sun et al. (2016b)). Treatment with sodium butyrate has been shown to exert many positive effects including protection from cell death, disease associated histone deacetylation, reduced symptoms of stroke, and improved learning and memory in models of Alzheimer's Disease (Bourassa et al., 2016). Cognitive symptoms of diseases and disorders including depression, brain ischemia, Alzheimer's disease, anxiety, Huntington's disease, bipolar disorder, and more have all been improved in animal models by treatment with sodium butyrate (Stilling et al., 2016, Bourassa et al. (2016)). While many genes are affected by histone deacetylation, the neurotrophic factors including BDNF and GNDF have bene found particularly sensitive to butyrate (Barichello et al., 2014). To summarize Stilling et al., (Stilling et al., 2016) butyrate has been shown to be effective at: facilitating long-term potentiation (Lattal et al., 2007, Vecsey et al. (2007)), converting short- to long-term memory (Haettig et al., 2011, Intlekofer et al. (2013)), and emulating environmental enrichment (Fischer et al., 2007). As local protein synthesis (and first transcription) at the synapse is required to create long-term memory or convert short- to long-term memory, butyrate may facilitate this process by inhibiting deacetylation and allowing transcription to occur (Stilling et al., 2016).

Short chain fatty acids are known to modulate the permeability of intestinal endothelial tight junctions (Peng et al., 2007) as well as blood-brain-barrier (BBB) integrity (Braniste et al., 2014). Prenatal development of the BBB is sensitive to maternal gut microbiome status, as fetal brain from germ-free (GF) mothers displays less expression of the tight junction protein occludin than those from pathogen-free mothers. Given the fetus does not have its own established microbiome, the mechanism is likely communicated humorally via the mother.

Braniste et al., (Braniste et al., 2014) demonstrated that GF mice have increased BBB permeability in adulthood using three different methods. Positron emission tomography imaging revealed greater uptake of a BBB impermeable tracer in GF vs pathogen-free mice. Evans blue tracer was detected in blood vessels of adult pathogen-free mice, whereas it was also detected in the brain parenchyma of adult GF mice, demonstrating leakage across the BBB. Leakage of Evans blue tracer in GF mice was similar to pathogen-free mice treated with tumor necrosis factor α (TNF-α), highlighting that the leakage was substantial. Finally, anti-NMDA receptor reactive antibody induced cell death revealed abnormal cell morphology in the CA1 region of the hippocampus of GF animals, morphology not present in pathogen-free animals. Germ-free mice also had reduced cell numbers in the same region, an effect not observed in pathogen free animals as there was no transport of the anti-NMDA antibody across the BBB. Two-photon microscopy revealed

that impaired BBB integrity was not related to lack of pericyte coverage or reductions in vascular density. Instead, adult GF mice displayed reduced expression of the tight junction proteins occludin and claudin-5 but not zona occludens (ZO-1) in the frontal cortex, striatum, and hippocampus, an effect similar to that seen in fetal brain from GF mothers.

Colonization of GF mice with flora from pathogen-free mice decreased permeability closer to that of pathogen-free mice and differentially increased expression of endothelial tight junction proteins in the frontal cortex, hippocampus, and striatum. As fetal brain displayed impaired BBB but fetuses lack a gut microbiome, the investigators tested whether this effect was related to SCFA, which could circulate to the fetus from the mother. Adult GF mice treated with *C. tyrobutyricum* or *B. thetaiotaomicron* for two weeks, or sodium butyrate for 72 hours showed reduced permeability such that no dye was detected outside blood vessels of the frontal cortex, striatum, or hippocampus. Western blot analysis revealed GF mice treated with sodium butyrate had increased expression of occludin in the frontal cortex and hippocampus. This work demonstrates that the integrity of the BBB is sensitive to gut microbial status beginning during fetal development and continues to be impaired into adulthood. Integrity was not impaired due to structural differences in vascular density or pericyte coverage, but rather the presence of tight junction proteins. Treatment with probiotics or sodium butyrate restored integrity, suggesting that the mechanism is linked to SCFA.

1.3.4 Gut-brain-axis and the serotonergic system

As a precursor to serotonin, tryptophan plays an important role in the serotonergic system (Botting, 1995). Serotonin is among the first neurotransmitter system to develop in mammals (Rho and Storey, 2001), and the concentration, number of uptake sites, and receptor binding of serotonin is higher in early life compared to adulthood (Murrin et al., 2007). As tryptophan becomes the 1st limiting amino acid in infant metabolism when the protein concentration is lowered (Lönnerdal, 2014), fluctuations in tryptophan availability may impact the development of the serotonergic system.

Evidence from Clarke et al., (Clarke et al., 2013) demonstrates that GBA regulation of the hippocampal serotonergic system is sex-specific. Germ-free and conventionally colonized (CC) male and female mice were raised for 6-9 weeks and measures of the serotonergic system, stress response, and immune response were taken. Afterwards, a separate group of male GF mice were conventionalized at weaning by group-housing in conventional cages with bedding and fecal material from CC mice. The authors found that regardless of sex, GF mice displayed greater corticosterone release in response to stress and a reduced expression of TNF-α in response to *ex vivo* splenocyte stimulation with lipopolysaccharide, demonstrating increased response to stress but a reduced reaction to inflammation that was independent of sex. Sex differences arose in the hippocampus however, as male GF mice had decreased BDNF, increased serotonin, and increased 5-HIAA, a metabolite of serotonin, in the hippocampus compared to CC controls. Female GF and CC had similar

expression of BDNF, serotonin, and 5-HIAA. In plasma, male GF mice displayed elevated tryptophan and a reduced kynurenine:tryptophan ratio. It is estimated that only 1% of peripheral tryptophan is used for serotonin synthesis, whereas more than 95% is used for synthesis of kynurenines (Botting, 1995). Here, the amount of tryptophan used was shunted towards greater synthesis of serotonin and reduced synthesis of kynurenine. Interestingly, female GF and CC mice did not show any difference in plasma tryptophan, however female GF mice also had a reduced kynurenine:tryptophan ratio. This study provided the first evidence that alterations in the microbiota produce sex-specific changes in the hippocampal serotonergic system.

Upon recolonization at weaning, adult colonized GF mice continued to display elevated serotonin and 5-HIAA levels, demonstrating that the serotonergic system was programmed in early neonatal life to display elevated serotonin. As serotonin decreases with aging, elevations of serotonin in GF animals may be an indication of immaturity. Despite having elevated serotonin, recolonization of GF mice restored plasma tryptophan and the kynurenine:tryptophan ratio to CC levels. Additionally, recolonization lowered the number of transitions between light and dark compartments in the light/dark box, a measure of increased anxiety, to CC levels. While an increase in anxiety may appear adverse, this study replicated previous findings where GF animals display less anxiety than CC controls (Sampson and Mazmanian, 2015). Together these data suggest that even when the availability of the serotonin precursor tryptophan is brought to CC levels, hippocampal elevations in serotonin persist.

Multiple studies have confirmed perturbations to this system. Peripherally, GF mice display reduced serotonin in serum (Wikoff et al., 2009, Yano et al. (2015)), plasma (Yano et al., 2015), colonic contents (Yano et al., 2015), and feces (Yano et al., 2015), increased tryptophan in serum and plasma (Clarke et al., 2013, Wikoff et al. (2009)), and a reduced kynurenine:tryptophan ratio (Clarke et al., 2013). In a metabolomics study of colonic luminal samples, GF mice displayed higher levels of all amino acids except tryptophan, which was lower in comparison to controls (Matsumoto et al., 2012), indicating that although all amino acids are altered, tryptophan is selectively decreased. Centrally, GF mice display increased serotonin and its metabolite 5-HIAA in the hippocampus (Clarke et al., 2013), as well as elevated turnover of noradrenaline, dopamine, and serotonin in the striatum (Heijtz et al., 2011). In contrast, a study in rats showed the opposite trend of GF mice, as GF rats had reduced serotonin in the hippocampus when compared to specific-pathogen-free (SPF) controls (Crumeyrolle-Arias et al., 2014). Converging evidence demonstrates that there is a significant perturbation of the serotonergic system in GF animals, however given differences between animal models the relationship is unclear. Tryptophan is seemingly at the center of metabolism, however modulating tryptophan does not always reverse serotonergic phenotypes in GF models. Germ-free models are not the only evidence that the microbiome has a significant impact on serotonin. *L. Helveticus* provided to adult rats in a model of hyperammonemia decreased serotonin metabolism and improved cognitive and anxiety-like behavior (Luo et al., 2014) and *B. infantis* treatment increased plasma

tryptophan and kynurenine while reducing 5-HIAA in the frontal cortex. This effect of 5-HIAA is opposite of that observed by Clarke et al., (Clarke et al., 2013) where 5-HIAA was increased in GF models, suggesting a possible mirroring of phenotypes between GF and probiotic models.

1.3.5 Vagal Communication

Forsythe, Beinenstock, & Kunze (Forsythe et al., 2014) provide a thorough review on vagally mediated GBA communication. The vagus nerve is the tenth cranial nerve, containing both motor and sensory neurons, and acts as the primary afferent pathway for organs in the abdominal cavity to the brain (Forsythe et al., 2014). They innervate the intestine in a 9:1 afferent:efferent ratio, where they project to the muscular and mucosal layers, but not the intestinal lumen (Forsythe et al., 2014). Thus, ligands in the lumen must be absorbed and reach vagal afferent endings intact, or act indirectly through a second messenger such as cholecystokinin, serotonin, or gut peptides (Li and Li, 2007).

Little is known regarding the nature of VFA on vagal afferent neurons (VANs). It is known in the rat that mesenteric afferent nerves in the jejunum can be evoked by both short and long chain fatty acids (Lal et al., 2001). While long chain fatty acids (e.g., oleate) active cholecystokinin (CCK) receptors on enteroendocrine cells which secrete CCK into the mucosa where CCK can act on VANs, butyrate can stimulate VANs directly (Lal et al., 2001). In addition to stimulating VANs independently of CCK, this effect is not mediated by other neurotransmitters such as serotonin. In other words, butyrate acts as a ligand for VANs and is not communicated through a second messenger. The authors state that since no vagal fibers penetrate into the lumen, it is unlikely that SCFA in the lumen act on vagal fibers, they are likely absorbed and would act on VANs in the lamina propria (Lal et al., 2001). The effects of butyrate on VANs were abolished with subdiaphragmatic vagotomy, suggesting that the effect of butyrate on mesenteric afferent neurons is specific to VANs (Lal et al., 2001). However, this data is specific to the small intestine, and not the colon.

The idea that the vagus nerve plays a role in modulating behavior is not new. The vagus nerve mediates behavioral responses to cytokines (Luheshi et al., 2000, Krahl et al. (2004), Konsman et al. (2000)), indicating behavioral adaption in the presence of immune insults requires the vagus nerve. Stimulation of the vagus nerve can lead to an anxiolytic and anti-depressive effect in rats (Krahl et al., 2004), however this effect is not always replicated (Biggio et al., 2009). Animals infected with *Campylobacter jejuni*, which colonizes the cecum (Goehler et al., 2005), induces an anxiogenic effect (Lyte et al., 1998). On the other hand, non-pathogenic bacteria demonstrating probiotic effects such as *L. rhamnosus* have been shown to have vagal dependent anxiolytic effects in mice concomitant with alterations to GABA receptor gene expression in the amygdala, hippocampus, and cortex (Bravo et al., 2011). As discussed previously, Vazquez et al., (Vazquez et al., 2016) report increases in cognition in response to 2'fucosyllactose supplementation that is vagal-dependent. Together these data suggest that the vagus nerve is required for both prebiotics and probiotics to exert beneficial effects on behavior.

1.3.6 GBA and cognition function

A review by Sampson & Mazmanian (Sampson and Mazmanian, 2015) details the impact of probiotics or a lack of microbiota on anxiety and emotion in both humans and animals. Treatment of humans with *L. helveticus* R0052 and *B. longum* reduces self-reported anxiety (Messaoudi et al., 2011a). In a human trial, subjects provided a multispecies probiotic containing *bifidobacteria* and *lactobacilli* reported less aggressive thoughts, which contributed to reduced cognitive reactivity to sad moods (Steenbergen et al., 2015a). Another study conducted in humans found that consumption of a probiotic containing fermented milk product resulted in reduced action in functional networks containing the insula, periaqueductal gray, and somatosensory cortex. These results were interpreted as evidence that the probiotic product reduced responsivity to emotional stimuli (Tillisch et al., 2013). Evidence for decreased anxiety or depression-like behavior in mice provided probiotics in GF mice is replicated in several studies (Bravo et al., 2012, Clarke et al. (2013), Desbonnet et al. (2010), Heijtz et al. (2011), Neufeld et al. (2011), Savignac et al. (2014), Savignac et al. (2015), Selkrig et al. (2014)). These actions have been observed simultaneously as serotonergic alterations, but there has currently not been a conclusive mechanistic link between the two. Although some studies have shown increased anxiety-like behavior in GF mice (Bercik et al., 2011) and stress-sensitive rats (Crumeyrolle-Arias et al., 2014), the majority of work points to a decrease in anxiety or depression-like behaviors.

In addition to modulating anxiety and depression, there is evidence to support increases in cognitive performance. Consumption of high fiber diets in children is positively associated with better performance on an attentional inhibition task (Khan et al., 2015), and there have been several reports of prebiotics increasing cognition in various models (Kao et al., 2016). These may be related to production of SCFA, which as mentioned have shown to be protective in various neurodegenerative diseases (Bourassa et al., 2016). Interestingly, a link has been proposed between SCFA and autism, as direct injections of propionate into the brain are associated with abnormal social behaviors (Macfabe, 2012). Care should be taken when interpreting these results however, as experiments are conducted using higher than physiological doses of SCFA that reach the brain without being filtered through the blood-brain-barrier first. Nevertheless, a positive association has been found for aggression and cecal lactic acid and volatile fatty acids (Hanstock et al., 2004), and an inverse association between plasma lactate and recognition memory (Hanstock et al., 2010). The relationship between SCFA and cognition remains unclear, as effects of SCFA are usually concurrent with stimulation of the microbiome.

1.3.7 The GBA in neonatal models

While there has been a proliferation of research on the GBA in adult models, our understanding of the relationship in early life is lacking. However, the same effects of perturbations/stimulations of the GBA that exist in adults seem to also exist in the neonate. As discussed previously, it was demonstrated that

supplementation with GOS increases expression of the NMDA receptor NR1 in the frontal cortex, NR2 in the hippocampus, and BDNF mRNA in the dentate gyrus of the hippocampus (Savignac et al., 2013). Similarly, the same prebiotic provided to neonatal rats increased BDNF, synaptophysin, and the NMDAR subunit GluN2A at postnatal day 22, which persisted after supplementation ended up to PND56 (Williams et al., 2016).

While stimulation of the microbiota with a prebiotic may causes changes that persist into adulthood, a recent study demonstrated that GF animals pass on their anxiolytic phenotype to conventionalized offspring. Diaz Heijtz et al., (Heijtz et al., 2011) demonstrated that adult GF mice display reduced anxiety-like behavior and increased motor activity compared to SPF controls, elevated turnover of noradrenaline, dopamine, and serotonin in the striatum, and higher expression of synaptophysin and PSD-95 in the striatum. When GF mice were conventionalized, their offspring displayed similar locomotor activity as SPF mice, however their anxiety-like behavior remained similar to their GF paternal counterparts. Expression of synaptophysin and PSD-95 were however reduced to similar levels as SPF controls in the striatum. Although the locomotor and synaptic protein expression were rescued by conventionalization, the decreased anxiety phenotype observed in GF mice was passed down to conventionalized offspring. These data show that the microbiota can alter behavior not only of the host animal, but of offspring as well.

As mentioned previously, fetal integrity of the blood brain barrier is impaired when the mother is raised GF, and GF animals continue to display impaired BBB integrity into adulthood (Braniste et al., 2014). Additionally, Clarke et al., (Clarke et al., 2013) demonstrated that recolonization of GF mice at weaning does not rescue GF induced abnormalities in the serotonergic system. Together, these data highlight the importance of establishing a healthy microbiome even before birth, and insults to the microbiome can have permanent effects.

1.3.8 Conclusions

The gut-brain-axis is intricately linked both directly via the vagus nerve and indirectly through several humoral signaling routes. Perturbations of this communication appear to have effects that can last into adulthood or even transfer to future offspring. As the serotonergic system is one of the first neurotransmitter systems to develop, early life is a critical window wherein establishment of the infant gut microbiome is crucial. Stimulation of the microbiota with either prebiotics or probiotics have shown promise in enhancing cognitive function. Seemingly, most prebiotics are capable of enhancing cognition, however future work should control for dosage, timing, and mode of delivery to disentangle the effects of prebiotic stimulation versus ingredient specificity.

1.4 Use of the pig as a biomedical model

1.4.1 Introduction

The pig has been well documented and accepted as a translatable model for research in cardiovascular systems (Stanton and Mersmann, 1986), gastrointestinal systems (Yen, 2001), nutrition (Odle et al., 2014), diabetes (Larsen and Rolin, 2004), toxicology (Lehmann, 1998), and surgical techniques (Richer et al., 1998). However, its use in the neurosciences has been slowly adopted or completely absent at many research institutions. Despite the dearth of research using the pig as a model for human brain and cognitive development, its use in psychology and brain development began 50-65 years ago (Moore and Marcuse, 1945, Dickerson and Dobbing (1966)). At that point, the piglet was primarily used for species comparative research. Since then, use of the pig in neuroscience-related fields has grown.

1.4.2 Brain and Cognitive Development in the Pig

Gross neuroanatomy and structural development of the pig brain is strikingly similar to that of the human. On a total volume basis, one month of growth in the pig appears nearly equivalent to one week of growth in the infant, with the pig brain 10% the size of the human brain (Knickmeyer et al., 2008, Conrad et al. (2012a)). Rate of growth as compared to adult brain weight demonstrates that both pigs and humans have a perinatal growth spurt (Dobbing and Sands, 1979). Monkeys, sheep, and guinea pigs have a prenatal growth spurt whereas rats and rabbits exhibit a postnatal growth spurt. An analysis based on brain weight alone belies the complexity of development, as others have shown biphasic growth curves when assessing lipid content of the brain. As a percent of total lipids, phospholipids steadily decrease from late gestation into adulthood, whereas cerebrosides (monoglycosylceramides, present in higher concentrations in white matter than grey matter) increase in both the cerebrum and spinal cord (Sweasey et al., 1976). The proportion of cholesterol to total lipids remains relatively constant throughout this same time period. Notably, there appear to be two large "spurts" of growth that occur. Cerebroside content appears to peak two weeks prior to birth and three weeks after birth. Furthermore, reports of the fatty acid composition of the brain demonstrate the pig has similar composition to that of humans (Purvis et al., 1982). Beyond brain weight, the sexually dimoprhic rate of growth of several regions have been identified (Conrad et al., 2012a). Despite these advancements, there is still little understanding of the rate of cellular and molecular development regarding neurogenesis, dendritogenesis, and synaptic proliferation and pruning. Still, other reviews have established our understanding of pig neurobiology is advanced enough to study neurotransmitter systems (Lind et al., 2007) and psychiatric disorders (Gieling et al., 2011b).

A distinct advantage of the pig over other animal models stems from the precocial nature of the species. The pig's sensorimotor system is developed enough that it can perform behavioral tasks designed to assess discrimination, spatial learning and memory, and problem-solving skills shortly after birth (Friess et al.,

2007, Siegford et al. (2008), Wang et al. (2007b)). Although a relatively young field, there have been some reviews that have synthesized the amount of research conducted in the behavioral and cognitive fields using the pig (Gieling et al., 2011b, Gieling et al. (2011a), Kornum and Knudsen (2011), Lind et al. (2007)). Though the pig has a relatively well-developed sensorimotor system at birth, it is important to understand the capabilities and limitations of its sensory system when designing behavioral tasks. Little research has been conducted investigating the limits of the adult pig senses, and much less investigating the limits of the developing pig, indicating a need for more research in the ontogeny of the pig sensorimotor system. The following section will briefly discuss the critical sensory abilities of the pig that should be considered when developing a behavioral task.

1.4.2.1 Visual and auditory development

Female domestic pigs between 21 and 42 days of age are able to visually discriminate between blue and gray colors, but not red from gray, or green from gray, suggesting that pigs are only able to discriminate blue among the primary colors (Tanida et al., 1991). When discriminating between objects, 4-month-old female domestic pigs appear to have difficulty distinguishing small symbols at close ranges, and their visual acuity is lower than that of cattle and humans (Zonderland et al., 2008). Additionally, evidence suggests 2.5-3-year-old male minipig barrows learn olfactory discrimination tasks faster than visual discrimination tasks (Croney et al., 2003, Lind et al. (2007)). Tanida and Nagano (Tanida and Nagano, 1998) discovered that for 8-week-old Göttingen minipigs, visual and auditory cues are more important than olfactory cues in allowing pigs to discriminate between people. In general, there has been very little research cataloguing the visual capabilities of the pig, and these studies are spread across different breeds, age, and sex of pigs. It is unknown why for some discrimination tasks olfactory is the dominant sense used, whereas for others visual and auditory cues are predominantly used. Understanding the sensory modality most preferred between discrimination tasks is critical for designing robust behavioral tasks, especially when such tasks are to be used with piglets at extremely young ages.

The auditory capacity of the domestic pig was first demonstrated using a creative application of classical and operant conditioning (Heffner and Heffner, 1990). Three 4-month-old water-deprived pigs (Durock, Hampshire, and Yorkshire genetics) were trained to maintain contact with a waterspout using their mouths in order to receive water. A 2-second tone was then presented, followed by a mild shock. The shock was adjusted for each animal to be at the lowest level that would provide an aversive stimulus, but not instill a fear of the spout in the animals. The shock was accompanied by a light that cued the presence of the shock and when it was safe to return to the spout. Using this paradigm, the pigs learned to break contact with the spout upon hearing the 2-second tone. This method was used to determine that pigs can sense auditory signals from 42-40,500 Hz, and they have the greatest sensitivity of signals between 250 and 16,000 Hz, as compared with humans, who have an auditory threshold of 31-17,600 Hz, and greatest sensitivity around 4,000 Hz (Heffner and Heffner, 1992). Beyond pitch, domestic pigs can recognize a variety of vocalizations

that are used to send information about sender identity and arousal state (Held et al., 2009). It has also been shown that novel noises are aversive to pigs (Hutson et al., 2000, Talling et al. (1998)), however when no immediate threat is recognized, pigs can quickly habituate to the novel sound. This may have implications for the use of artificial or white noise during behavioral tests. As mentioned previously, pigs use auditory cues along with visual cues, to discriminate between people (Tanida and Nagano, 1998), even though neither are as developed as the pig's olfactory system.

1.4.2.2 Olfaction and gustation

Neuroanatomy of the pig suggests a highly developed olfactory system (Lind et al., 2007), however there have been relatively few studies investigating olfaction in pigs. Pigs are famous for being used to hunt truffles (Pacioni, 1986), and olfactory discrimination and sensitivity of the pig surpasses that of primates (Kristensen et al., 2001, Meese et al. (1975)). As mentioned earlier, Croney et al. (Croney et al., 2003) demonstrated that 2.5-3-year-old minipigs can complete multiple choice discrimination tasks using either olfactory or visual cues, and though performance was not dependent on type of cue, pigs trended towards better performance when using olfactory cues. Sommerville and Broom (Sommerville and Broom, 1998) stated that olfaction is the most important sense for transmission of social information between pigs. They note that pigs may be responsive, but not necessarily aware of the presence of primer pheromones (pheromones that have a delayed effect on reproductive physiology). However, pigs do appear to be responsive and aware of releaser pheromones (pheromones that affect behavior of an animal immediately). Olfactory information is sent via the individual's odor spectrum (scent) and pheromones, which may be influenced by sexual state (Signoret et al., 1975), social identity (Croney et al., 2003, Meese et al. (1975), Held et al. (2002)), and aggressive disposition (McGlone, 1990). Much research centered on pheromones in the pig is related to pubertal or post-pubertal development, not early development. It has been shown, however, that for human infants, dogs cannot tell identical twins from each other until they are weaned, at which point diet may play a role in establishing an individual's odor spectrum. The influence of diet on odor spectrum may be common across species, and this may be important to acknowledge when studying social development or social cognition of the piglet.

Pigs aged 5-7 weeks can sense flavors that humans perceive as bitter, sour, sweet, and salty (Jones et al., 2000, Perry (1992)). Blair and Fitzsimmons (Blair and Fitzsimmons, 1970) demonstrated that pigs had complete aversion to food when an extremely bitter compound was added. However, following ingestion of small quantities of the bitter compound by food-deprived pigs, food intake returned to normal, demonstrating that flavor preference, as indicated by food intake, is dependent on a fed vs. food-deprived state. Held et al. (Held et al., 2009) noted that methods to test sensitivity to flavor include measuring food consumption (Baldwin, 1976), using preference tests (Mclaughlin et al., 1983), or using operant procedures (Arave, 1996). Pigs may have inherent tendencies toward flavors that indicate high levels of macronutrients (Kyriazakis et al., 1990), and it has been demonstrated using a progressive ratio task that pigs are more highly motivated

to work for solutions with the greater sucrose concentrations (Kennedy and Baldwin, 1972). Glaser et al. (Glaser et al., 2000) showed that pigs avoid bitter tastes while preferring carbohydrate solutions to water, and Nofre et al. (Nofre et al., 2002) demonstrated that two compounds rated by humans as the sweetest (e.g., guanidinoacetic acid derivatives) are also the most preferred compounds by pigs. Food reward is commonly used as a positive reinforcement in operant conditioning, so effort should be put forth to make sure the food reward is an adequate reinforcer. A variety of foods have been used as reinforcers, such as chocolate milk (Elmore et al., 2013), sweetened water (Kennedy and Baldwin, 1972), raisins (Tanida and Nagano, 1998), and even dog biscuits (Croney et al., 2003).

1.4.3 Behavioral Assessment Methods Used in Human and Animal Infant Research

In order to use the pig as a model for human infant cognition, it is imperative to understand research and methods used to assess infant cognition. As infants are preverbal, understanding the methods used to assess them can provide guidance when designing studies for piglets. In this section, several methods used in infant research that have potential for translation in the piglet model will be discussed. They broadly fall into the categories of novelty preference paradigms, conditioning paradigms, and deferred imitation.

1.4.3.1 Novelty Preference Paradigms

Novelty preference paradigms make use of the tendency for infants to look longer at novel or complex stimuli. The visual paired comparison (VPC) tasks and variants thereof have been used to assess infant recognition of images or scenes since the mid 1980's (Baillargeon et al., 1985). The premise of the task consists of exposing an infant to a pair of identical stimuli (or scene), a delay, and another pair of stimuli with one stimulus from the first trial and a novel stimulus. The task can be modified to assess recognition of previously seen stimuli or to investigate an infant's understanding of reality. In the study of learning and memory, recognition of the old stimulus is indicated when the infant demonstrates looking time greater than 50% (chance) towards the novel stimulus. By varying the delay, researchers can test the retention length of recognition memory (Rovee-Collier and Cuevas, 2009). This technique can be altered to include habituation, where an infant is repeatedly shown a stimulus, and habituation is demonstrated when looking time at that stimulus falls to a set criterion (such as 50% of the looking time on the first few trials).

Using habituation, research suggests infants demonstrate retention for about 5-10 seconds at 3-4 months, 1 minute at 6 months, and 10 minutes at 9-12 months of age (Rose et al., 2007). However, when the stimuli are achromatic images, retention is much longer. With multiple sessions of training at 5 months, infants exhibit retention up to 48 hours (Fagan, 1973). Recognition for faces is also greater, as newborns can exhibit retention of a face for 2 minutes (Pascalis and de Schonen, 1994), and 3-month-olds exhibit retention for up to 24 hours (Pascalis et al., 1998). The dramatic increase in retention length for faces indicates that the

brain regions for processing faces may be more developed than for processing non-facial stimuli. Not only is the length of retention important, but also the speed at which infants habituate to stimuli. Differences in habituation rate are associated with processing speed and later recognition memory. Short lookers (i.e., those that habituate quickly) have better recognition memory than long lookers (i.e., those that habituate slowly) (Rovee-Collier and Cuevas, 2009). Attention may mediate habituation rate, thus affecting recognition memory. Rose et al. (Rose et al., 2007) demonstrated that when 5-month-old infants categorized as long lookers had enhanced attention to aspects of a target stimulus, looking time was shortened and recognition was improved to the point that long lookers were indistinguishable from short lookers.

The visual paired comparison task was originally developed to assess visual development in primates in the 1950's (Fantz, 1956). It also presents a tool to study recognition memory, which requires inputs from the hippocampal region, dentate gyrus, subicular complex, and adjacent cortical areas (Nemanic et al., 2004). There have been several other tasks used to assess recognition memory in primates and rodents. The delayed nonmatching-to-sample (DNMS) task is similar to the VPC task but rather than passively observe stimuli the subject manually interacts with the stimulus, and is rewarded based on exploration (or the choice to explore) the familiar rather than novel stimuli, which is opposite of most subjects' natural preference for novelty. Both the VPC and DNMS require the perirhinal cortex, but only VPC is dependent on the hippocampus (Nemanic et al., 2004). Behaviorally, both tasks require familiarization with an item, and then a choice phase where the familiar item is presented with a novel stimulus. Because DNMS involves active participation, performance relies on item familiarity, whereas the passive participation in the VPC task relies on both item familiarity and event recollection.

The DNMS task has seen extensive use in primates, as well as rodents (Clark et al., 2001). Studies in monkeys have shown that this task shows differential performance across development, indicating that it is a model task to assess ontogeny of the behavior and underlying brain regions (Stanton, 1992). Nielsen et al. (Nielsen et al., 2009) developed the task for 12-14-month-old male Göttingen minipigs. The paradigm was validated in pigs by altering delay interval and administering scopolamine, an anti-cholinergic drug that has been shown to impair performance on the DNMS task in other animal models. The authors noted that pigs performed similarly to macaque monkeys, but unlike rodents, did not show a natural tendency to alternate their choice. However, this is in contrast to research from Laughlin and Mendl (Laughlin and Mendl, 2000) demonstrating that pigs do have a natural tendency to spontaneously alternate choices.

Other paradigms that make use of the natural preference to look at or engage with novel stimuli to test recognition memory include novel object recognition. Novel object recognition has had long-standing use in rodent research (Ennaceur and Delacour, 1988). In this paradigm, an animal is allowed to explore one or two identical objects, and a delay is presented before the second trial where the animal is allowed to explore a familiar object from trial one and a completely novel object. Recognition of the old object is inferred if the animal spends more time exploring the novel object. Because this paradigm uses exploration as an indicator

of recognition, the animal must have the motor capacity to explore and thus demonstrate recognition. This task can be used to assess function of the medial temporal lobe as well as the prefrontal cortex (Antunes and Biala, 2012).

The novel object recognition task has been adapted for use in 12-14-month-old Göttingen pigs [Kornum et al. (2007), Moustgaard et al. (2002)) and 42-day-old domestic pigs (Gifford et al., 2007) as the spontaneous object recognition task, and in pigs as young as 3 weeks old (Fleming and Dilger, 2017). A benefit of the novel object recognition task is that it does not require repeated trials or use of rewards and punishments. This allows for much greater throughput, as it takes less time for the animal to complete the task. Because larger animals can be difficult to work with, increasing the throughput capability of a behavioral task is of great importance. However, a significant limitation of novelty preference paradigms are their binary output. Subjects, both human and animal, may display behavior that can be quantified by continuous variables, however typically the ultimate output is a "yes" or "no" criterion to determine if recognition memory was present. While true improvements to the development of cognition or brain development may have occurred, performance will have reached a ceiling if subjects already demonstrated recognition memory previously.

1.4.3.2 Conditioning Paradigms

One of the constraints in studying infant memory is that human infants are preverbal, and after infancy has passed and language has been acquired, humans have great difficulty recalling memories from infancy. It is for this reason that development of long-term memory was first shown using young animals, and not human infants. Using conditioning paradigms similar to those used with animal models, infant researchers have been able to assess the ontogeny of long-term memory, as noted in a review of infant memory development (Campbell and Coulter, 1976). In general, conditioning paradigms fall into two categories: operant and classical (or Pavlonian).

Classical conditioning is a paradigm in which a subject is presented with a neutral stimulus (the conditioned stimulus, CS) paired with a biologically salient stimulus (the unconditioned stimulus, US). The US causes an unconditioned response (UR), and after repeated pairings of the CS and US, the CS subsequently causes a conditioned response (CR) that is the same as the UR. In the classic case of Pavlov's dogs, the ringing of a bell (i.e., the CS) was paired with food (i.e., the US), and the food stimulated salivation (i.e., the UR). After repeated pairings of the bell and food, the bell was ultimately able to stimulate salivation (i.e., the CR) (Rudy, 2008).

Operant conditioning, famously developed and popularized by B.F. Skinner (Skinner, 1938), refers to the use of reinforcement or punishment to either increase or decrease a target behavior. To increase a behavior, any reward can be applied (i.e., positive reinforcement) or an aversive stimulus can be removed (i.e., negative reinforcement). To decrease a behavior, an aversive stimulus can be applied (i.e., positive punishment) or a reward can be removed (i.e., negative punishment). Infant research uses mostly positive reinforcement,

whereas animal research uses a variety of reinforcement and punishment strategies.

1.4.3.3 Operant Conditioning

For 2-6-month-old infants, the mobile conjugate reinforcement task entailed connecting infant's leg to a mobile with a ribbon. Over a timed period, the infant is trained to associate movement of its leg with movement of the mobile (i.e. when the infant kicks the mobile moves). This can be used to assess whether or not an infant remembers interacting with a mobile. Retention of such a memory can be tested by measuring baseline extent of kicking, providing a delay, and returning the infant to the mobile to re-assess kicking rate. If kicking rate is above baseline, retention of the memory is inferred, as further reviewed by Rovee-Collier and Cuevas (Rovee-Collier and Cuevas, 2009). Hartshorn et al. (Hartshorn et al., 1998) found that 2-month-old infants exhibit retention for 1 day, 9-month-olds exhibit retention for 6 weeks, and 18-month-olds exhibit retention for 13 weeks. For infants 6-24 months old, the operant train task is used. Instead of moving a mobile, the infant temporarily moves a train around a track by pressing a button. Hartshorn and Rove-Collier (Hartshorn et al., 1997) proved that this task is equivalent to the mobile conjugate task by demonstrating retention at 6 months is the same between tasks.

Due to limited mobility, operant tasks for the infant may be much more limited than those for the pig, rodent, or primate. For the piglet, any operant task chosen should be sensitive to development and appropriate for the cognitive capability of the age in question. A variety of operant tasks have already been developed for the pig, including the 8-arm radial maze (Dilger and Johnson, 2010), T-maze (Elmore et al., 2013), avoidance conditioning (Kratzer, 1969), a conditional go-no-go task (Moustgaard et al., 2005), and many others. For a comprehensive review see Gieling et al. (Gieling et al., 2011a).

1.4.3.4 Deferred Imitation

In the deferred imitation paradigm, a researcher models a target action (e.g., sticking their tongue out of their mouth), and after a delay, an infant is given an opportunity to repeat the target action. This procedure can also be altered such that verbal prompts are given during models and infants are allowed to imitate immediately (known as elicited imitation). One of the key differences between deferred imitation and operant tasks is that there is no training involved and infant participation is largely passive. This is most likely the reason why infants do not exhibit retention in deferred imitation tasks as long as they do in operant tasks. It has been demonstrated that infants as young as 6 months can show imitation (Barr et al., 1996). Similar to mobile and train operant tasks, length of retention of deferred imitation increases linearly from 1 day long for 6-month-old infants, to 4 weeks long for 18-month-old infants (Hayne, 2007).

Imitation as a form of social learning may be an invaluable survival skill for pigs. Under laboratory conditions, pigs may not need to observe others foraging for food, as it is obtained by the pig with little to no work. However, in natural environments, piglets learn about food as they forage with the sow and littermates

(Held et al., 2009). Nicol and Pope (Nicol and Pope, 1994) demonstrated that pigs that had watched a trained littermate feed from one of three troughs tended to feed from the same trough the trained littermate had originally chosen. Held et al. (Held et al., 2000) took advantage of this phenomenon and created the informed forager paradigm. In this paradigm, pigs are trained to search for food hidden in one of eight possible locations in a foraging arena. After training, pigs foraged in pairs consisting of a dominant and a subordinate individual, where only the subordinate knew of the food location. Over several trials of foraging together, the naïve pigs learned to follow the trained subordinates to the hidden food, rather than search randomly as they had previously done. This suggests pigs have some ability to compare foraging efficiency between individuals.

The informed forager paradigm also showed that subordinate pigs changed their foraging strategy to avoid exploitation by dominant pigs by leading them away from baited buckets or changing behavior to arrive at the baited bucket before the dominant pig. It is noted that these behaviors were the same as those in monkeys, which exhibit conditioned tactical deception (Held et al., 2009). These behaviors are deceptive tactics in reference to the consequence of such behaviors, not necessarily the mental state of the pigs exhibiting deceptive behaviors. Further investigation into whether or not a pig would alter foraging behavior based on the visual perspective of others was also completed. Held et al., (Held et al., 2009) hypothesized that when pigs cannot see where food is hidden, they will follow an individual that was able to see where the food was hidden.

1.4.3.5 Behavioral assessment and development of attention in infants

Measuring attention in infancy is similar to the VPC task mentioned earlier. The infant is provided a stimulus, and the duration and latency of looking at the stimulus is measured as an indicator of attention. The amount of time spent looking varies by age, sharply increasing from 2-8 weeks of age, dropping to a trough from 8-20 weeks of age, and gradually increasing throughout the first year of life (Colombo et al., 2004). Generally, look time is understood as an indicator of processing speed (Colombo et al., 1991, Colombo and Mitchell (1990)) and is related to better cognitive outcomes in later development, however only modestly so (Colombo et al., 2004). During the first year, there is a negative correlation between later outcomes on IQ and language with look time (Colombo, 1993, McCall and Carriger (1993), Tamis-LeMonda and Bornstein (1989)), the shorter the look time, the better the performance. However, past the first year, longer look durations predict better performance in later outcomes (Lawson and Ruff, 2004b, Lawson and Ruff (2004a), Ruff and Lawson (1990)). These differences are thought to be due to increases in information processing during the first year, resulting in decreased look duration. Whereas increased ability to sustain attention on an object past the first year leads to an increase in look duration.

Stimuli infants can remember or are familiar with tend be attended to less than novel stimuli. In this way, attention and memory have a reciprocal relationship. Infants aged 9-10 months but not 6.5 months old have

a greater tendency to turn to a distractor when examining a familiar toy rather than a novel toy (Oakes et al., 2002). In other words, the strength of a memory trace has an effect on the distribution of attention. Colombo and Cheatham (Colombo and Cheatham, 2006) argue that the coinciding development of both memory and attention during the end of the first year form the basis for the development of more complex, high order skills that develop during late infancy and toddlerhood.

To our knowledge, there has been no development of a task specifically designed to assess attention in piglets. The closest has been the development of a go/no-go task by Moustgaard and colleagues (Moustgaard et al., 2005) to assess associative learning. Three common tasks used to assess attention in rodents are the multiple-choice serial reaction time task, signal detection task, and attentional set-shifting tasks (Bushnell and Strupp, 2009). Challenges to developing tasks similar to these are the appropriateness of operant tasks for young piglets, many operant tasks are more suitable for older animals. Using look duration and latencies as described in Colombo and Cheatham (Colombo and Cheatham, 2006) may also be more difficult in an animal than a human. However, methods such as novel object recognition and the DNMS task require attention to be successfully completed, and time spent physically exploring an object may be analogous to look time in infants

1.4.4 Experimental Design with a Large Animal Model in Nutritional Neuroscience

The benefit of using the piglet as a preclinical model derives from its similarities in neuroanatomy and development. The pig is similar to the human as it has a gyrencephalic brain and exhibits a perinatal growth spurt, as compared to rodents which have lissencephalic brains and experience postnatal growth spurts (Dobbing and Sands, 1979, Sauleau et al. (2009)). Given these advantages, the piglet is more costly to feed and house, and the molecular techniques available to use in rodent animal models overshadow those available for porcine research. Additionally, due to its similarities to humans, there are greater ethical concerns to consider when working with the piglet, and public perception of research with the pig should not be underestimated. Increasing the longitudinal power and designing experiments to allow correlational analysis are two simple techniques that take advantage of the previous benefits while optimizing the amount of data that can be collected from a single experimental unit. Due to the size of the piglet brain, greater sample can be collected. The piglet hippocampus is roughly two to three times the length of the entire mouse brain. Where it may be difficult to collect enough rodent brain tissue to conduct multiple analyses on the same target, piglet tissue is usually present in high enough quantities that the same tissue can be homogenized and used for analytical and molecular techniques. While reducing the number of animals needed for an experiment, correlational and predictive statistical analyses can then be performed on subjects that contain data from all outcomes. At the same time as providing preclinical data, this increases the power to make plausible mechanistic statements. This contrasts with smaller animals, wherein multiple separate

groups of animals may be needed to quantify expression of protein, mRNA, or metabolites, thus precluding the ability make statements about the relationships between outcomes.

Whenever possible, longitudinal designs should be employed. This can be done by extending the length of a trial or by increasing the frequency at which data is collected. Many biomedical facilities are not equipped to rear piglets from their 1-2 kg birthweights to their older 50-100+ kg counterparts. To counter this, including weekly or monthly measures into an existing timeline can provide longitudinal power. While the piglet develops more slowly than the rodent, behavioral differences in recognition memory can be observed even after a single week of growth (Fleming and Dilger, 2017). Measures of brain tissue can only be collected at one time, and this has prompted a proliferation in development of neuroimaging techniques to assess piglet brain development (Mudd and Dilger, 2017). In the absence of such techniques, collecting blood, urine, and/or feces as peripheral biomarkers of nutrient status throughout the course of the trial is advantageous. These can be combined with omics approaches to capture extremely large datasets. Although expensive and technically challenging, the use of omics level techniques allows for wide breadth of screening and combining multiple omics techniques can allow in-depth statistical analysis.

As mentioned previously, being able to compare experimental outcomes with normative data increases the reliability of nutritional claims. The use of longitudinal designs and appropriate controls by their nature provide normative data. If the experimental unit is artificially reared, experimental objectives should include sow-reared controls if possible, similar to the use of breast-fed controls in infant research. While there are challenges to interpreting differences between sow-reared and artificially reared animals due to the differences in diet and social interaction, the inclusion of a sow-reared group allows for greater collection of normative data and can thus bring greater power to a nutritional claim.

Wainright & Colombo (Wainwright and Colombo, 2006) detail various scenarios wherein cross-sectional data can either exaggerate or underplay the importance of a nutritional intervention. Assuming a cognitive function matures linearly and that performance on a task measuring that function is accurate, there are multiple ways a nutritional intervention may alter performance. A nutritional intervention may increase performance at an early age, yet performance becomes equivalent between treatment groups at a later age. The danger in this scenario may be in the overinterpretation of a cognitive benefit at earlier ages, when after maturity there is no measurable increase in performance. This may be a preferable alternative to the opposite scenario, in which a nutritional intervention has no impact on early developing functions but is revealed to have increased cognitive performance in later development. If longitudinal data is not collected experimenters may encounter the first scenario whereupon early performance is increased, which may or may not hold true throughout development. Conversely, if they do not collect data from later ages, it may be concluded that a nutritional intervention had no effect, when differences would have arisen were the study to be continued.

In addition to dynamic changes in cognitive function throughout development, Wainwright and Colombo

(Wainwright and Colombo, 2006) note that it is possible for a nutritional intervention to enhance the function of one cognitive domain at the expense of another. Damage to the hippocampus can improve performance on stimulus-response based tasks, whereas damage to the caudate nucleus can increase performance on spatial learning tasks. While lesions or damage to specific regions may be a more exaggerated manipulation than nutritional supplementation or deficiency, the same concept applies. This phenomenon suggests that capturing cognitive performance using multiple behavioral tasks is superior to using a single task. This has been observed in our own research, as supplementation with polydextrose and galactooligosaccharide was shown to have no impact on spatial recognition memory or response to restraint stress but did improve object recognition memory (Fleming et al., 2017). This is likely related to the specificity of function of each brain region. For example, the perirhinal cortex is required to remember what object was seen before in an object recognition task but is not required to remember where an object was seen before, that function is delegated to the hippocampus (Barker and Warburton, 2011). Thus, object recognition memory is not the same as spatial recognition memory. The evidence that a nutrient does or does not improve object recognition memory does not prove or disprove its ability to modulate spatial recognition memory.

1.5 Conclusions

Pediatric nutrition is a highly regulated field and optimizing the health of the infant is a challenging task. Behind every claim, sufficient data must exist to support a nutrients safety and efficacy. Establishing the appropriate controls, normative data, and sensitive and accurate behavioral tasks to investigate the impact of nutrition on cognition is crucial. The impact of nutrition and cognition may be subtle, as such it is important to capture the development of a range of cognitive domains over time. This comes with logistical obstacles, however there are methods of overcoming these. The piglet model holds a unique position in that it is one of few species that can bridge the gap between rodent, primate, and human research. Though not as well established as other models, the pig has been used for decades to study animal welfare, and its use in neuroscience is quickly growing. Thus far, methods that use operant conditioning have been most established in the pig, whereas methods that rely on classical conditioning and novelty preference are lacking. Operant conditioning can be extremely labor intensive and time consuming, whereas novelty preference paradigms have the advantage of requiring fewer trials, thereby increasing throughput. Pairing the pig's similar neuroanatomy with behavioral methodologies analogous to that used in infant research creates a powerful model for investigating the impact of early life nutrition on brain and cognitive development.

Chapter 2

Young pigs exhibit differential exploratory behavior during novelty preference tasks in response to age, sex, and delay[1]

2.1 Abstract

Novelty preference paradigms have been widely used to study recognition memory and its neural substrates. The piglet model continues to advance the study of neurodevelopment, and as such, tasks that use novelty preference will serve especially useful due to their translatable nature to humans. However, there has been little use of this behavioral paradigm in the pig, and previous studies using the novel object recognition paradigm in piglets have yielded inconsistent results. The current study was conducted to determine if piglets were capable of displaying a novelty preference. Herein a series of experiments were conducted using novel object recognition or location in 3- and 4-week-old piglets. In the novel object recognition task, piglets were able to discriminate between novel and sample objects after delays of 2 min, 1 h, 1 day, and 2 days (all $p < 0.039$) at both ages. Performance was sex-dependent, as females could perform both 1- and 2-day delays ($p < 0.036$) and males could perform the 2-day delay ($p = 0.008$) but not the 1-day delay ($p = 0.347$). Furthermore, 4-week-old piglets and females tended to exhibit greater exploratory behavior compared with males. Such performance did not extend to novel location recognition tasks, as piglets were only able to

[1]Fleming, S.A., and Dilger, R.N. 2017. Young pigs exhibit differential exploratory behavior during novelty preference tasks in response to age, sex, and delay, *Behavioural Brain Research. 321.* 50–60. The copyright owner has provided permission to reprint

discriminate between novel and sample locations after a short delay ($p > 0.046$). In conclusion, this study determined that piglets are able to perform the novel object and location recognition tasks at 3-to-4 weeks of age, however performance was dependent on sex, age, and delay.

2.2 Introduction

The piglet is increasingly being used as an animal model to investigate brain trauma (Duberstein et al., 2014, Sullivan et al. (2013), Browne et al. (2011)), neuroscience (Lind et al., 2007, Gieling et al. (2011b)), animal welfare (Held et al., 2009), pediatric nutrition (Liu et al., 2014, Rytych et al. (2012), Getty and Dilger (2015)), and toxicology (Nunoya et al., 2007). The piglet is much larger than the rodent, and large sample sizes require relatively greater effort to acquire data compared with rodents. As such, there is a need to develop behavioral tests that are accurate, timely, and require relatively little labor. Novelty preference paradigms have been widely used in humans as well as primate and rodent models to assess recognition memory, spatial memory, discrimination, and other cognitive domains (Clark et al., 2001, Nemanic et al. (2004), Rovee-Collier and Cuevas (2009), Antunes and Biala (2012), Westbrook et al. (2014)). Specifically, these paradigms have been extensively used in infant research to assess development of memory and perceptual abilities in infants (Pascalis and de Schonen, 1994, Pascalis et al. (1998), Rose et al. (2004), Rose et al. (2007), Baillargeon and DeVos (1991)). Thus, the use of such paradigms in animals imparts greater translational value to infant models than most operant based maze training tasks, which are the predominant tasks used. The novel object recognition (NOR) and novel location recognition (NLR) tasks require no operant training and can be used repeatedly on the same experimental unit, making them efficient behavioral tasks to assess recognition memory (Antunes and Biala, 2012). Robustly establishing replicable methods to use these tasks will become increasingly necessary as the piglet model gains greater acceptance within the field of neuroscience.

Although there has been some investigation into piglets' spontaneous preference for novel or familiar objects (Wood-Gush and Vestergaard, 1991), using novel objects as enrichment (Van de Weerd et al., 2003), or exploration of novel objects (Wood-Gush et al., 1990, Hemsworth et al. (1996)), to our knowledge, only three experiments have formally investigated NOR in piglets (Moustgaard et al., 2002, Gifford et al. (2007), Kornum et al. (2007)), and no experiments have investigated NLR. Research using 12–14-month-old Göttingen minipig boars (i.e., mature, intact male pigs) suggested pigs can remember objects for short delays of 10 min or 1 h but were unable to do so at a longer delay of 24 h (Moustgaard et al., 2002, Kornum et al. (2007)). However, 5-week-old domestic pigs were shown to be capable of remembering objects for longer delays of 3 h or 5 days, but not for a shorter delay of 1 h (Gifford et al., 2007). Such differences may be accounted for by a range of different genetics, rearing conditions, and testing methodology. These conflicting results have led to the belief that the task may be unsuitable for the pig, and a recent review states that the NOR task "has not yet indisputably proven its relevance in pigs" (Gieling et al., 2011a). Understanding the basic ability of the piglet to perform novelty preference tasks will be crucial in advancing

both the field of piglet behavior and cognition, and additionally those fields seeking to use the piglet as a model for investigation.

The present study involves compilation of three individual experiments designed to test the hypothesis that young pigs are capable of displaying a novelty preference at a range of short and long delays in an NOR task, and that performance is modulated by sex and age. Additionally, we tested the hypothesis that piglets are capable of displaying a novelty preference in the NLR task as previous literature has suggested piglets can complete spatial tasks such as the associative 8-arm radial maze and a spatial T-maze (Dilger and Johnson, 2010, Elmore et al. (2013)).

2.3 Methods

All animal care and experimental procedures were in compliance with National Research Council Guide for the Care and Use of Laboratory Animal Care and Use Committee and approved by the University of Illinois Urbana-Champaign Institutional Animal Care and Use Committee.

2.3.1 Experiment 1:3- vs. 4-week-old piglets on NOR

The aim of experiment 1 was to replicate previous research to determine if piglets were capable of displaying recognition memory of objects observed after a range of delays, and how performance changed with age. Piglets were tested at weeks 3 and 4 of life on the NOR task using delays of 2 min, 1 h, and 1 day.

2.3.1.1 Animals

Twenty domestic piglets ($n = 13$ female, $n = 7$ male) from three litters were received from the University of Illinois Imported Swine Research Laboratory on postnatal day (PND) 2. Piglets were placed in an artificial rearing system in caging units that allowed them to see, hear, and smell, but not touch, neighboring piglets to prevent competition for food and control individual cage environments. All animals were given access to supplemental water and provided nutritionally complete milk replacer formula. Piglets were weighed individually each day and fed 285, 305, and 310 ml/kg body weight (BW) on PND 2–4, 5–10, and 11–40, respectively, with five meals of equal volume provided each day starting at 0700 h and ending at 2000 h. Lights went on at 0700 h and off at 1930 h. Piglets were observed at each feeding and given health scores at 0700 h and 1830 h to track any weight loss, vomiting, diarrhea, or lethargic behavior. Four piglets were removed from study due to failure to thrive.

2.3.1.2 Behavior

Piglets were tested on the NOR paradigm at postnatal weeks (PNW) 3 (PND 17–21, $n = 13$) and 4 (PND 24–28, $n = 12$) using three different delays: 2 min, 1 h, and 1 day. Order of delay was randomized and

counterbalanced across all subjects. Object pairs 1–3 were used for PNW 3 and object pairs 4–6 were used for PNW 4 (Figure 2.1). Object pair and position of the novel object (north or south quadrant of the arena) were also counterbalanced. Piglets were given three 10-min habituation trials over the course of three days leading up to the sample trials wherein they were allowed to explore the empty arena. Piglets were then given 5 min to explore two identical objects during the sample trial, and then 5 min to explore one of the sample objects from the sample trial plus a novel object during the test trial (see supplemental video at teh journal for an example of a test trial). Piglets were placed into the arena at the west end facing east, equidistant between the two objects that were secured to the floor. The objects and arena were sprayed with water to remove urine and feces between individual trials. All testing occurred during the light cycle and after piglets had received their first meal of milk replacer for the day. By study completion, piglets were tested on 3 habituation trials and all delays at PNW 3 and 4 for a total of 6 habituation trials and 6 delay tests for each subject.

The arena used was approximately 2.13 m $\times$ 2.44 m with age- and species-appropriate, plasticol-coated, 9 gauge expanded metal flooring (Tenderfoot^{TM}, Tandem Products, Minneapolis, MN). Walls were constructed of black high-density polyethylene panels reinforced with a heavy-duty wire-filled gate. A digital camcorder (Samsung, Seoul, South Korea) was mounted overhead to capture approximately 1.22 m $\times$ 1.83 m of the arena, centered over the objects. Objects used for all experiments were screwed into metal plates and clipped to hooks in the center of the arena, preventing objects from being removed while allowing small movements when manually touched to encourage exploration.

A preliminary control test ($n = 4$) was conducted to assess if piglets had an innate preference for individual objects. The same methods as described above were used for testing except the sample trial was omitted, and after habituation trials piglets were given a one trial test containing the object pairs used for test trials. Due to small sample size this test was omitted from analysis and a subsequent test was conducted using piglets from experiment 2.

2.3.1.3 Analysis and statistics

Videos from all experiments were analyzed using a commercially-available software package (Ethovision XT 11®, Noldus Information Technology, Wageningen, The Netherlands). Time spent investigating objects was recorded manually by mapping start and stop conditions to specific keys on a computer keyboard. Experimenters were blind to all treatment conditions during analysis. Investigations were classified as nose directed behavior such as rooting, mouthing, or sniffing towards the objects within approximately 15.24 cm. Rubbing up against, standing over, or standing near objects were not counted as investigations.

All statistical analyses were conducted using SAS Enterprise Guide© 5.1 (SAS Institute Inc., Cary, NC). Although the recognition index (or variants thereof) are of primary interest to those using the NOR/NLR task, several other measures were included as measures of general exploratory behavior to provide greater

insight to piglet behavior. Measures analyzed included recognition index (time spent investigating novel object/time spent investigating all objects), total visit time (total time spent investigating all objects), mean visit time (mean length of exploration during a single investigation), number of object visits, latency to first visit, and habituation towards objects (change in total visit time/change in time). All measures were also analyzed for both sample and novel objects individually.

Exclusion criteria for all experiments was applied by first removing any trials incorrectly performed due to experimental error (sample trials, $n = 2$; test trials, $n = 2$), removing any trials that used objects for which piglets expressed an innate preference (sample trials, $n = 23$; test trials, $n = 22$), removing non-compliant piglets (as defined by investigation of either objects for less than 2 s; sample trials, $n = 1$), and lastly by removing outliers that exceeded a studentized residual with absolute value of 3 for each measure analyzed. Piglets who were non-compliant in the sample trial were removed from analysis in the test trials. Two pairs of objects were excluded from analysis: pair 6 due to being insecurely attached to the floor during testing, and pair 3 because control tests revealed piglets had an innate preference for the object used as a novel stimulus (testing procedure described in Section 2.3.2.2. Preference was categorized as on average, unequal time spent exploring objects used as sample and novel object.

All measures were analyzed via mixed model, repeated-measures ANOVA to determine main effects of age and delay, as well as the age x delay interaction. Litter and sex were included as random effects in the model. When measures failed to meet homogeneity of variance they were appropriately transformed using either the square root or natural log or analyzed via Friedman test. To assess whether piglets were capable of demonstrating a novelty preference (i.e., recognition index greater than 0.50), one-tailed t-tests were conducted at each age by delay group comparing their recognition index to a null preference score of 0.50. Non-normal variables were analyzed via sign test, and all tests were conducted with an $\alpha = 0.05$. Only significant effects are reported, for full results see supplemental tables at the journal article (Fleming and Dilger, 2017).

2.3.1.4 Inter-observer reliability

All experiments were analyzed by a single experimenter. Two blind experimenters analyzed a subset of trials ($n = 20$) to assess inter-observer reliability. Correlation analysis suggested high agreement (all $r > 0.993$, $p < 0.001$) and a paired t-test revealed no significant differences between observers (all $p > 0.741$).

2.3.2 Experiment 2: male vs. female piglets on NOR

Experiment 2 was conducted to both replicate and extend results from experiment 1. A low sample size and unbalanced design precluded an analysis of sex effects in experiment 1. The aim of experiment 2 was to determine if exploration of novel objects differed by sex, and if piglets could display a novelty preference at a delay longer than 1 day. Additionally, the thigmotaxic behavior of piglets was analyzed to determine if

piglets behave similarly to rodents in an open field.

2.3.2.1 Animals

Thirty-six domestic piglets ($n = 19$ female, $n = 17$ male) from five litters were received from the University of Illinois Imported Swine Research Laboratory on PND 2 and stratified to two delay groups ($n = 18$/delay) according to litter, birth weight, and sex (19 female, 17 male). Piglets were reared as described in Section 2.3.1.1 and milk replacer was provided at 500 ml/kg BW per day. Considering the higher rate of milk replacer provision, no supplemental water was provided as dosing formulations contained sufficient water to sustain the piglets. Piglets were hand-fed six times a day with first and last daily meals provided at 0700 h and 2200 h, respectively.

2.3.2.2 Behavior

Piglets were tested on the NOR paradigm during PNW 3 (PND 18–21) using either a 1- or 2-day delay. Testing was performed as described in Section 2.3.1.2. Prior to testing, piglets were given two 10-min habituation trials over two days. Habituation was reduced from three to two trials as by subjective accounts piglets appeared to lay or sit down and cease exploratory behavior by the end of the third habituation trial in Experiment 1. For test trials, all piglets were counterbalanced and randomly assigned 1 of 2 object pairs (either pairs 1 or 5). Post-testing, all piglets were given two one-trial control tests, using 2 of the remaining 4 object pairs that remained novel, to test for innate preferences. If on average piglets spent more time with one of the objects than the other in a pair, piglets were defined as having an innate preference for that object. By study conclusion, each piglet had undergone 2 habituation trials, either a 1- or 2-day delay paradigm, and 2 control trials. Position of the novel object was counterbalanced across all trials.

The arena used was 1.52 m $\times$ 1.22 m with age- and species-appropriate black plastic flooring (Hog Nursery Slat, Double L Group©, Dyersville, IA) raised on a metal frame to allow to feces and urine to drop below and wash away during inter-trial cleaning. Walls were constructed of the same material as in Section 2.3.1.2. A video camera (Basler acA1300-300gc camera; Basler AG, Ahrensburg, Germany) was mounted overhead that captured the entire arena area.

2.3.2.3 Analysis and statistics

Analysis was conducted using the same methods described in Section 2.3.1.3. A mixed model ANOVA was conducted to assess main effects of sex, delay, and interaction effects of sex by delay with litter as a random effect. Measures analyzed include those described in Section 2.3.1.3, as well as distance moved, rate of distance moved, percent time spent in the perimeter of the arena, and percent time spent in the center of the arena. Trials that included experimental error (control trials, $n = 3$; test trials, $n = 2$), non-compliant piglets (sample trials, $n = 4$; test trials, $n = 3$) and outliers were removed prior to analysis. A paired t-test

was also conducted to determine if there was a difference in time spent in the perimeter and center as of the arena determined by a mean difference different from zero as a measure of thigmotaxic behavior.

2.3.3 Experiment 3: NLR in 4-week-old piglets

The aim of experiment 3 was to determine whether 4-week-old piglets were capable of displaying recognition memory for spatial arrangements of objects previously encountered. Additionally, similar to the NOR control tests, control tests were conducted to assess whether piglets have an innate preference for locations of objects within the arena.

2.3.3.1 Animals

Twenty male domestic piglets were received as part of a larger cohort from the University of Illinois Imported Swine Research Laboratory on PND 2 from eight litters, grouped in 4 replicates (n = 6, 6, 5, and 3 in replicates 1, 2, 3, and 4, respectively), and reared as described in Section 2.3.1.1. Milk replacer was dosed by an automatic feeding system that provided 285 or 325 ml/kg BW from PND 2–6 and 7–30, respectively, with 10 equal meals provided between 0700 h and 2000 h.

2.3.3.2 Behavior

Novel location recognition tests were conducted as described in Section 2.3.2.2 at PNW 4 (PND 25–31), however instead of switching objects in the test trials, the location of one of the sample objects was moved to a "novel" location. Additionally, two walls of the arena were taped with different colors and sizes of shapes to provide contextual cues. Piglets were tested on 2-min, 1-day, and 2-day delays in a randomized and counterbalanced order across replicates such that order of trial, objects used (i.e., pairs 1, 2, 4, and 5), and position of the novel location (locations 1, 2, 3, and 4) were all counterbalanced (Figure 2.2). Piglets from experiment 3 plus an additional group of piglets (n = 12) were tested on control trials similar to those described in experiment 2, however rather than testing for object preference, piglets were tested for preferences for the object locations used during test trials.

2.3.3.3 Analysis and statistics

Video was scored and measures analyzed as described in Section 2.3.1.3, however instead of tracking investigations of the novel and sample objects, investigations of the novel and sample locations were recorded. A mixed model repeated measures ANOVA was conducted to assess main effects of delay, with replicate included as a random effect. Trials that included experimental error (habituation, n = 2; sample, n = 3; test, n = 2), non-compliant piglets (sample trial, n = 2; test trial n = 5; control trial n = 3), and outliers were removed prior to analysis. Similar to objects removed for eliciting an innate preference in Section 2.3.1.3, location 2 was omitted from analysis for eliciting greater exploration of one of the locations (test trials, n = 20).

2.4 Results

2.4.1 Experiment 1:3- vs 4-week-old piglets on NOR

2.4.1.1 Control trials

One-tailed t-tests revealed that recognition indices for object pairs 1, 2, 4, and 5 were not different from a null preference of 0.50 (object pair 1: $n = 14$, mean = 0.446 ± 0.0552, $P = 0.345$; object pair 2: $n = 14$, mean = 0.424 ± 0.0587, $p = 0.217$; object pair 4: $n = 13$, mean = 0.524 ± 0.0523, $P = 0.658$; object pair 5: $n = 14$,mean = 0.491 ± 0.0446, $p = 0.847$) and a Sign test indicated object pair 3 was different from a null preference of 0.50 (object pair 3: $n = 12$, mean = 0.734 ± 0.0548, $p = 0.006$) (Figure 2.3A). The Friedman test revealed a main effect of object pair for mean novel object visit time (p= 0.003) (Figure 2.3B).

2.4.1.2 NOR trials

One sample t-tests revealed all groups had recognition indices greater than 0.50 (PNW 3, 2-min delay: $n = 8$, mean = 0.669 ± 0.0463, $p = 0.004$; PNW 3, 1-h delay: $n = 8$, mean = 0.656 ± 0.0654, $p = 0.025$; PNW 3, 1-day delay: $n = 8$, mean = 0.654 ± 0.0693, $p = 0.031$; PNW4, 2-min delay: $n = 7$, mean = 0.792 ± 0.0285, $p < 0.001$; PNW 4, 1-h delay: $n = 7$, mean = 0.720 ± 0.0574, $p = 0.004$; PNW 4, 1-day delay: $n = 9$, mean = 0.716 ± 0.0487, $p = 0.001$) (Figure 2.4A). Repeated measures mixed model ANOVA revealed a main effect of age for total visit time ($p = 0.002$) and mean total visit time (p= 0.001) during sample trials (Figure 2.4BC). During the test trial a main effect of age was observed for total visit time ($p < 0.001$), mean total visit time ($p = 0.0002$), novel visit time ($p < 0.001$), mean novel visit time ($p < 0.001$), and sample visit time ($p = 0.012$) (Figure 2.4D–H).

2.4.2 Experiment 2: male vs. female piglets on NOR

2.4.2.1 NOR trials

One-tailed t-tests indicated the recognition index was greater than 0.50 for females at both delays tested (1 day: $n = 8$, mean = 0.680 ± 0.0813, $p = 0.031$; 2 day: $n = 8$, mean = 0.627 ±0.0601, $p = 0.036$), and males for the 2-day delay ($n = 7$, mean = 0.652 ± 0.0458, $p = 0.008$), but not the 1-day delay ($n = 5$, mean = 0.546 ± 0.1158, $p = 0.357$) (Figure 2.5A). Mixed model ANOVA revealed no main effects of sex for any measure during the habituation or sample trials (all $p > 0.184$). A main effect of delay was observed during the test trial for percent time spent in the center of the arena ($p = 0.009$) (Figure 2.5B). A main of effect of sex was observed during the test trial for distance habituation ($p = 0.039$), number of novel visits ($p = 0.006$), and habituation towards the novel object ($p = 0.046$) (Figure 2.5C–E). Paired t-tests revealed piglets spent a greater amount of time in the perimeter of the arena than in the center of the arena during habituation trials ($p < 0.001$). Piglets spent an equal time in the perimeter and center of the arena during the sample trial ($p = 0.222$) and 1-day delay test trial ($p = 0.174$). During the 2-day delay test trial, piglets

spent more time in the center of the arena than in the perimeter ($p = 0.018$) (See Supplemental Table 2 and Supplemental Fig. 1 in Fleming and Dilger (Fleming and Dilger, 2017)).

2.4.3 Experiment 3: NLR in 4-week-old piglets

2.4.3.1 Control trials

One-tailed t-tests revealed that recognition indices for object locations 1, 3, and 4 were not different from a null preference of 0.50 (location 1: $n = 12$, mean = 0.456 ± 0.0750, $p = 0.570$; location 3: $n = 12$, mean = 0.413 ± 0.0652, $p = 0.209$; location 4: $n = 13$, mean = 0.475 ± 0.0620, $p = 0.695$), but object location 2 did differ from a null preference of 0.50 ($n = 7$, mean = 0.340 ± 0.0367, $p = 0.005$) (Figure 2.6A). Repeated measures mixed model ANOVA revealed a main effect of object location for mean total visit time ($p = 0.004$), mean novel visit time ($p = 0.011$), and mean sample visit time ($p = 0.010$) (Figure 2.6B–D).

2.4.3.2 NLR trials

One-tailed t-tests indicated recognition index was greater than 0.50 for the 2-min delay (2-min: $n = 13$, mean = 0.608 ± 0.0586, $p = 0.046$; 1-day: $n = 12$, mean = 0.493 ± 0.0666, $p = 0.543$; 2-day: $n = 14$, mean = 0.476 ± 0.0509, $p = 0.675$) (Figure 2.7A). Repeated measures mixed model ANOVA revealed a main effect of delay during the test trial for distance moved ($p = 0.021$), mean total visit time ($p = 0.018$), number of total visits (p= 0.019), sample visit time ($p = 0.034$), and mean sample visit time ($p = 0.012$) (Figure 2.7B–F).

2.5 Discussion

Novelty preference tasks will be pivotal for investigating piglet behavior due to the ease of execution and ability to modulate the design to the experimenters needs. Currently, there have only been three formal investigations into the piglet's ability to complete the NOR task, and these have yielded inconsistent results, partly due to differences in animals tested and methodology (Moustgaard et al., 2002, Gifford et al. (2007), Kornum et al. (2007)). The purpose of the present experiments was to identify the delays at which young piglets are capable of displaying a novelty preference using the NOR and NLR tasks, as well as provide additional data on the impact of sex and age on exploratory behavior. In general, female piglets displayed greater exploratory behavior than males, which was also true when comparing PNW 4 piglets to the same piglets at PNW 3. Young piglets displayed novelty preferences in the NOR task at a range of delays, but these were also sex dependent. Finally, piglets were only able to complete the shortest delay tested in the NLR task. Overall, these experiments highlighted a range of previously unknown factors that will be important to account for in future tests of piglet behavior and experimental design.

2.5.1 Experiment 1: 3- vs. 4-week-old piglets on NOR

Experiment 1 was conducted to assess whether piglets are capable of displaying a novelty preference at various delays, and if this ability is present during PNW 3 or 4. The piglet brain is rapidly developing during this time, thus we expected to see changes in behavior after a week of growth. Both males and females were used, however due to low power and unbalanced design sex was not included as a main effect. Results from this experiment suggest that by PNW 3 and 4 piglets exhibit recognition for objects at short (2 min), intermediate (1 h), and long (1 day) delays. Previous research suggests older piglets display a novelty preference at short delays but not long delays (Moustgaard et al., 2002, Kornum et al. (2007)), or long delays but not short delays (Gifford et al., 2007). Possible sources of disparity between results may arise from differences in methodology, breed, sex, and age of piglets used.

General exploratory behavior of older piglets differed from when pigs were tested at a younger age. At PNW 4, piglets displayed higher exploration times in total and per visit of both objects than at PNW 3 during the sample phase. During the test trial, piglets displayed greater exploration of the novel and sample objects at PNW 4 than PNW 3. From these data it can be concluded that older pigs exhibit generally greater exploratory behavior than their younger counterparts. Although order of delay and objects used were counterbalanced, the presence of testing effects may contribute to the observed effects.

Although the present data differ from what was previously observed, it is important to note that several methodological differences exist. In one study, 5-week-old domestic piglets were tested in pairs, which may have given rise to unintended social confounds (Gifford et al., 2007). Other groups used 1-year-old Göttingen minipigs (Moustgaard et al., 2002, Kornum et al. (2007)), and all groups fixed objects to the sides of their arena. In our study, objects were placed in the center of the arena and attached to metal plates loosely secured to the floor. This caused the objects to "rattle" slightly when touched, encouraging exploration by the pigs.

Additionally, object pairs that were omitted (pairs 3 and 6) from our study were subjectively the easiest objects to manipulate. The black tire and blue dustpan elicited greater levels of investigation, possibly because they were easily deformed compared to the other more rigid objects. However, after removing object pairs that elicited innate preferences from piglets, there was still a difference in exploratory behavior between object pairs. Mean novel visit time increased in a linear fashion for object pairs 1, 2, 4, and 5. This suggests that removing objects that elicit object preference may not be enough to omit variation due to the objects used. In such cases, rigorously testing objects prior to testing and counterbalancing objects across treatments is absolutely essential. These factors may further contribute to the differential results between groups. Anecdotally, it was noted that 2 habituation trials may be sufficient, because by the third trial, some piglets had laid down and ceased exploration by the end of the trial.

2.5.2 Experiment 2: male vs. female piglets on NOR

Experiment 2 was conducted to assess differences in exploratory behavior between males and females and test if piglets can display a novelty preference after a 2-day delay. Females were able to display a novelty preference at both the 1- and 2-day delays, whereas males were only able to display a novelty preference at the 2-day delay. This was a surprising result, as experiment 1 confirmed that piglets were capable of displaying a novelty preference at delays of 1-day or shorter. However, because sex was included as a random effect in experiment 1, it is plausible that participation by female subjects drove this effect. Females displayed a greater number of visits to the novel object and habituated to the novel object more quickly than males. As the trial progressed, males tended to decrease the amount of distance moved per minute, while females explored the same distance equally across each time bin. All piglets exhibited an increased proportion of time spent in the center of the arena during the 2-day delay than the 1-day delay. The reason why is not immediately clear. It may be that after 2 days, the objects are more "novel" as compared to after a 1-day delay, and the increase in novelty drove exploration in the center of the arena. However, the 2-day delay did not result in greater time spent exploring either object.

These data suggest that depending on sex, piglets are capable of displaying recognition of objects observed up to 2 days prior. These data are in contrast to that of previous work (Kornum et al., 2007), which suggests that piglets are only capable of displaying a novelty preference at a shorter delay (10 min), and in agreement with data supporting piglets' ability to remember objects at longer delays, such as delays ranging from 3 h to 5 days (Gifford et al., 2007). Most importantly, it demonstrates that males and females behave differently in response to novel stimuli, an effect that may be due to differential brain development. It is widely accepted that the perirhinal cortex is required to display recognition memory of objects in rodents (Balderas et al., 2008, Barbosa et al. (2013), Mendez et al. (2015), Norman and Eacott (2004), Barker and Warburton (2011)). To our knowledge there is no data on development of the perirhinal cortex in piglets, however research suggests that females reach peak growth rate of the hippocampus at approximately 3 weeks, whereas males reach this peak growth rate at 8 weeks of age (Conrad et al., 2012a). Furthermore, female pigs reach the age of maximum growth rate in the hippocampus, cerebellum, and brainstem before males. In general, the female piglet brain develops faster than the male, which may account for the differences observed in exploratory behavior and recognition memory in the current study. A secondary goal of this experiment was to assess whether piglets are similar to rodents in displaying thigmotaxic behavior in an arena (Drai et al., 2001). For analysis, the arena was split into 6×4 sections for a total of 24 sections, with the outer sixteen sections composing the "perimeter" while the inner eight sections composed the "center" of the arena. Piglets spent a greater proportion of time in the perimeter of the arena during the habituation trials (which also act as open field tests). Proportion of time spent in the center of the arena during the sample and 1-day delay were equal to that spent in the perimeter, while during the 2-day delay piglets spent more time in the center of the arena than the perimeter. These results suggest that piglets tend to behave similarly to rodents

when placed in an enclosed space. Reasons for spending more time in the perimeter may not be related to avoidance of open spaces as piglets appeared to be searching for an escape route, rather than displaying fear of the open area. Anecdotally, piglets spent a great deal of time rooting the juncture between floor and wall or the corners of the arena. As such, spending more time in the perimeter of the arena may be due to reward from tactile stimulation rather than avoidance of open spaces. Using a circular or rectangular arena with rounded corners may help reduce time spent in the corners and encourage exploration of the open area.

2.5.3 Experiment 3: NLR in 4-week-old piglets

The aim of experiment 3 was to investigate whether or not piglets could demonstrate a novelty preference in the NLR task at 4 weeks of age. To our knowledge, this is the first test of NLR in young pigs. Piglets were able to demonstrate a novelty preference after a 2-min, but not 1- or 2-day delay. It may be that at this age, although able to remember which objects were previously encountered, piglets are not capable of remembering the spatial arrangements of objects previously encountered for long periods of time. Although recognition index was relatively similar between trials, the mean total visit time, sample visit time, and mean sample visit time increased with delay duration (Figure 2.7C, E, and F). An increase in delay duration appears to increase exploratory behavior towards the sample object, perhaps indicating why no preference for novelty was observed after the 1- and 2-day delays. While these measures increase in a dose-response-like fashion, distance moved and the number of total visits was variable between delay durations (Figure 2.7B and D). Compared with results from NOR trials, these data suggest that young piglets are less adept at performing "where" based tasks than they are at performing "what" based tasks. This is in contrast to evidence from maze-based operant tasks, which have suggested that piglets are capable of completing spatial navigation tasks (Rytych et al., 2012, Elmore et al. (2013), Siegford et al. (2008), Hammell et al. (1975)). It is possible that in the absence of repeated training, piglets do not have the capacity to remember the spatial context after a single trial. As mentioned above, male piglets reach maximum growth rate of the hippocampus during PNW 8 (Conrad et al., 2012a), twice that of the age tested in this experiment, highlighting the relative immaturity of the piglet hippocampus at PNW 4. It is possible that relative immaturity of the piglet hippocampus is contributes to poor performance on the NLR task.

During the control trials piglets tested on location 2 had a recognition index different from 0.50 and tended to investigate the object in the sample location for a greater amount of time than the novel location; this may be due to the arrangement of the arena. The arena contained one door that pivoted to open. Upon opening, the object in the sample location was immediately visible, whereas the object in the novel location was not, possibly influencing exploratory behavior towards a preference for the immediately visible object. Additionally, piglets tended to explore the novel and sample objects in greater periods of time per visit if they were in location 1. Similarly to the control tests for NOR, this suggests that counterbalancing and rigorous testing of object locations is essential for the NLR task.

2.6 Conclusions

As established, previous research has yielded conflicting results using the NOR task in pigs (Moustgaard et al., 2002, Gifford et al. (2007), Kornum et al. (2007)). The aim of the present experiments was to replicate results from previous work and elucidate the effects of sex and age on performance of young pigs in the NOR task. Our data suggest that piglets were able to discriminate between novel and sample objects after delays of 2 min, 1 h, 24 h, and 48 h, but recognition of the novel object was dependent on sex. Females were able to display novelty preferences at both the 1- and 2-day delays, whereas males were unable to exhibit novelty preferences in the 24-h delay. Thus, sex and age played a significant role in the NOR task, as females and older pigs tended to exhibit greater exploratory behavior than males and younger pigs. Such performance, however, did not extend towards NLR tasks, as piglets were only able to discriminate between novel and sample location on the shortest delay tested.

A possible explanation for these results may lie in the differential development of the hippocampus in male versus female pigs. In rodents, the NOR task is dependent upon the perirhinal cortex (Norman and Eacott, 2004, Ennaceur et al. (1996)), whereas the NLR task is dependent upon the hippocampus (Barker and Warburton, 2011). The piglet hippocampus does not reach maximum volume until approximately 10.5 weeks in males and 7.4 weeks in females, and maximum growth rates of the hippocampus occurs at approximately 8.3 weeks in males and 3.3 weeks in females (Conrad et al., 2012b). Although there are currently no data on the growth of the perirhinal cortex in piglets, it may be that the accelerated growth of the female piglet brain as compared to the male piglet brain allows females to perform better on the NOR task. In the present study, no females were tested on the NLR task and all males were 4 weeks old, well under the age of either the maximum growth rate or peak volume of the hippocampus. This may be one explanation for the inability of young pigs to perform in the location tasks. Additionally, it may be that assessing spatial memory via spontaneous behavior is more difficult than using operant conditioning.

We propose that the NOR task is suitable for testing recognition memory in pigs at least 3 weeks old. Further testing should be conducted using the NLR task to assess the age at which piglets can complete longer delays, and if this coincides with maturation of the hippocampus. As use of the piglet as a translational model to study infant development continues, these tasks will may prove especially instrumental. Our analyses of exploratory behavior during control tasks demonstrate that thorough testing (including criteria other than recognition indices or time spent exploring the objects) of objects used prior to testing in conjunction with counterbalancing is absolutely critical. Furthermore, our data demonstrate that relevance of the NOR task may vary depending on sex and age in piglets, and such factors should be taken into consideration when using the NOR task to assess effects of experimental interventions.

2.7 Supplemental data

Supplementary data associated with this study can be found through the journal *Behavioral Brain Research* at http://dx.doi.org/10.1016/j.bbr.2016.12.027.

2.8 Figures

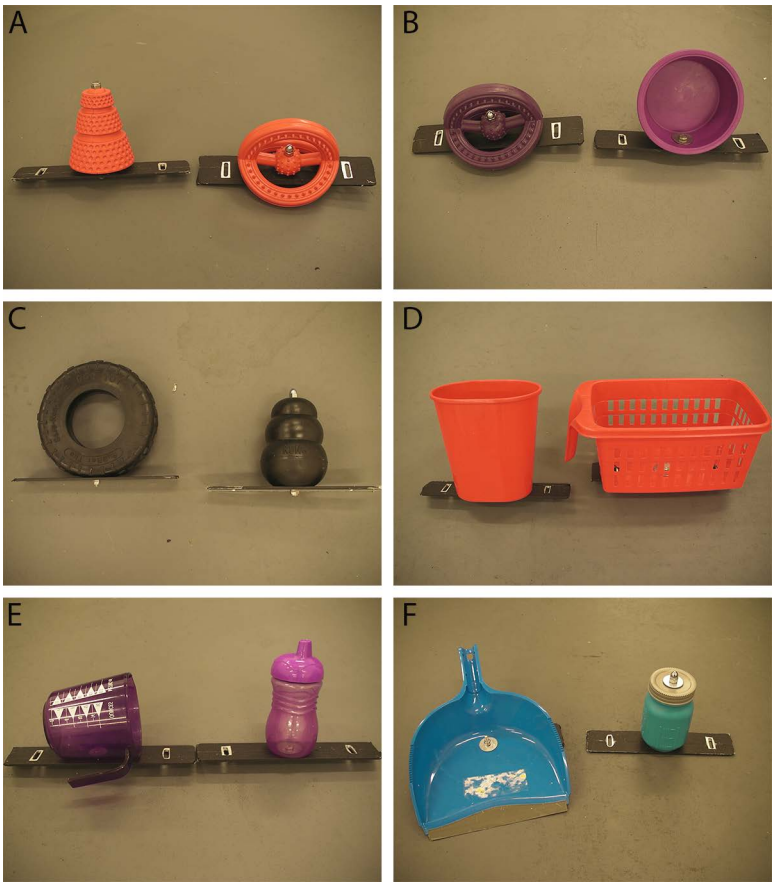

Figure 2.1: Objects used during the novel object recognition and novel location recognition tasks. Objects were chosen for similar color, size, texture, and durability, but not shape. In each picture, the familiar object is on the left and the novel object is on the right. **A)** Pair 1: orange rubber cone and orange rubber atom toy. **B)** Pair 2: purple rubber atom toy and purple plastic bowl. **C)** Pair 3: black rubber tire and black rubber Kong® ball. **D)** Pair 4: red plastic trash bin and red plastic basket. **E)** Pair 5: purple plastic measuring cup and purple plastic sippy cup. **F)** Pair 6: blue plastic dust pan and blue plastic mason jar.

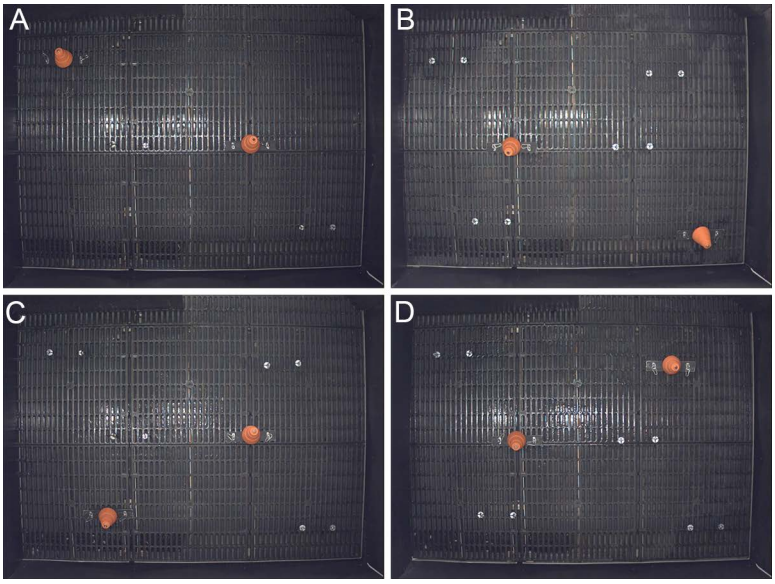

Figure 2.2: Locations used during the novel location recognition task. In each picture, the sample location is in the middle and the novel location is near a corner. During sample trials, both objects are placed in the middle. Two adjacent walls are marked a different shape to provide a visual cue (not pictured). **A)** Location 1. **B)** Location 2. **C)** Location 3. **D)** Location 4.

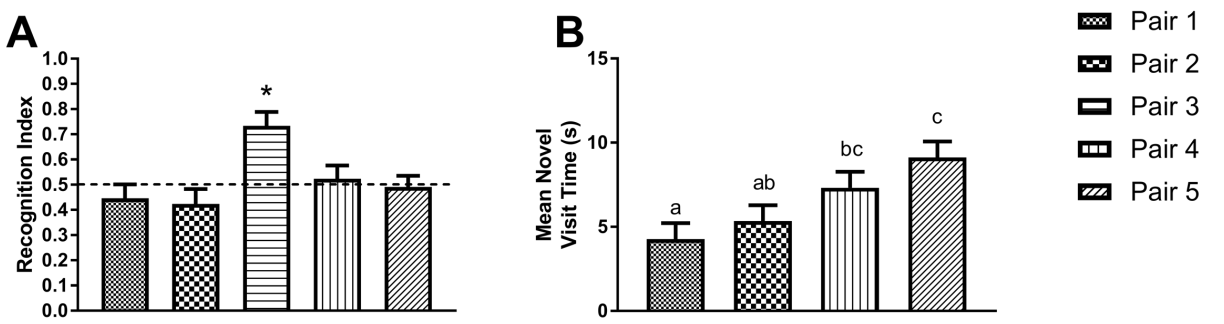

Figure 2.3: Effect of object pair on innate preference and exploratory behavior in a one trial control task. Animals were given a control task wherein they were exposed to the objects used as sample and novel stimuli without any previous sample trials. Pair 6 was omitted from all analyses due to being improperly fixed to the arena during testing. **A)** Recognition indices for object pairs 1-5. Note pair 3 had a recognition index score above 0.50 as determined by one-tailed t-test (marked by asterisk), indicating a preference for the object used as the novel stimulus. This pair was thus removed from subsequent analyses. **B)** Mean novel object visit time for pairs 1,2,4, and 5. Although these pairs were selected due to eliciting null preferences for recognition indices, the mean duration of time spent investigating the novel stimulus per visit in each pair was not equal. These data display the importance of counterbalancing objects used even after omitting object pairs that elicit innate preferences. All values are least square means ± pooled SEM (individual SEM reported for panel A). Significance between groups (indicated by letter superscript) determined by repeated measures mixed model ANOVA with Tukey adjustment.

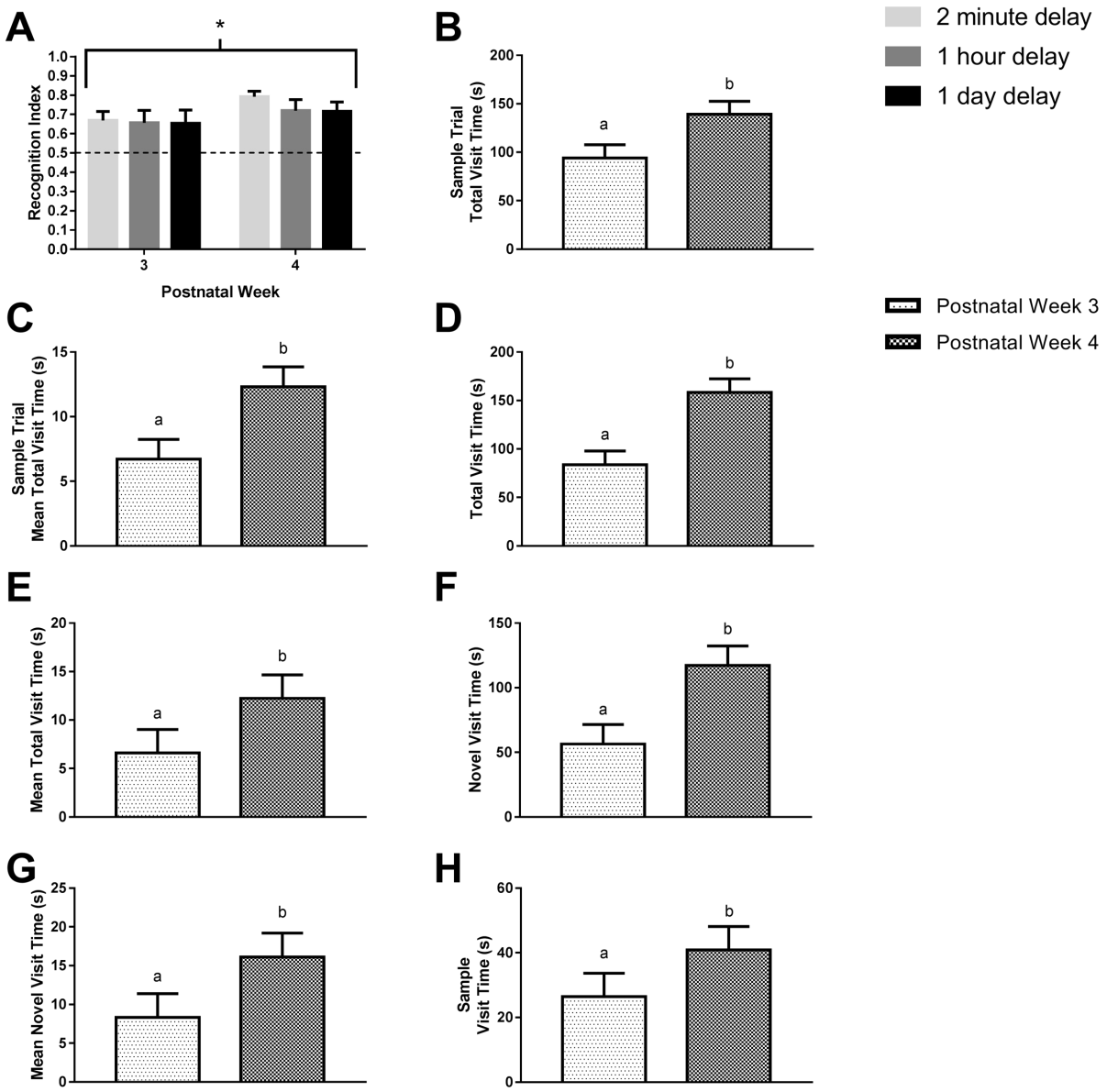

Figure 2.4: Effect of age and delay on exploratory behavior in pigs subjected to a novel object recognition task at postnatal weeks 3 and 4. **A)** Recognition indices for all delays tested at each age. Pigs exhibited a recognition index greater than 0.50 for all delays tested at each age as determined by one tailed t-test (marked by asterisk), however there were no differences between groups. Significance determined by one tailed t-test above a null preference score of 0.50. **B-E)** Piglets displayed increased time spent in total and per visit investigating both objects during both sample and test trials at postnatal week 4. **F-H)** At postnatal week 4 piglets displayed increased time visiting the novel object in total and per visit, and increased time investigating the sample object. All values are least square means ± pooled SEM (individual SEM reported for panel A). Significance between groups (indicated by letter superscript) determined by repeated measures mixed model ANOVA with Tukey adjustment.

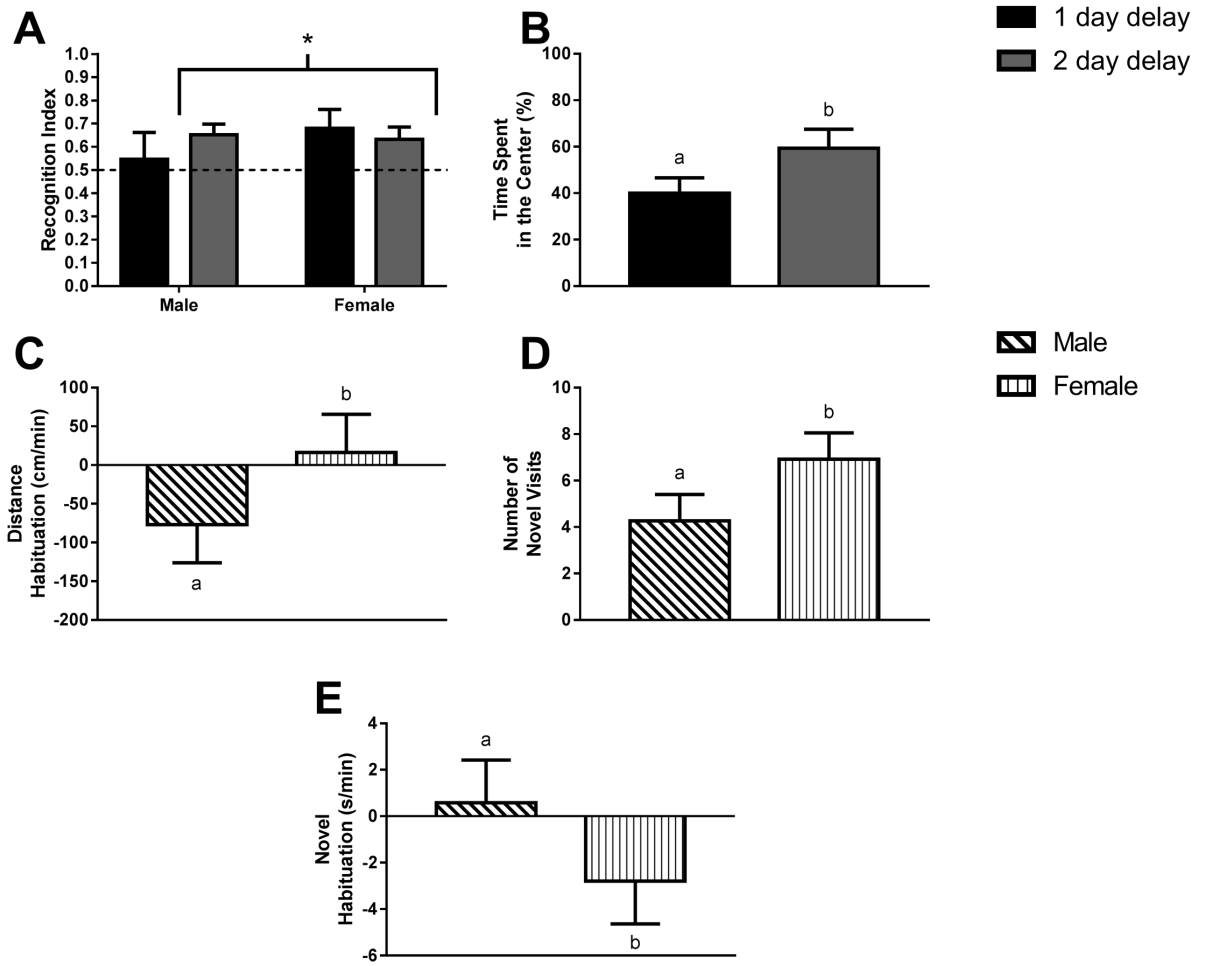

Figure 2.5: Effect of sex and delay on exploratory behavior in pigs subjected to a novel object recognition task at postnatal week 3. **A)** Recognition indices for each delay by sex. Female pigs exhibited a recognition index greater than 0.50 for all delays tested at each age as determined by one tailed t-test. Males were able to show a novelty preference at the 2-day delay, but not 1-day delay. There were no differences between groups. Significance determined by one tailed t-test above a null preference score of 0.50. **B)** Piglets displayed increased time spent in the center of the arena during after a 2-day delay as compared to a 1-day delay. **C)** Male pigs tended to decrease the distance moved each minute during the trial compared to female pigs. **D-E)** Female pigs displayed an increase number of visits to the novel object and habituated more quickly to the novel object compared to male pigs. All values are least square means ± pooled SEM (individual SEM reported for panel A). Significance between groups determined by mixed model ANOVA with Tukey adjustment.

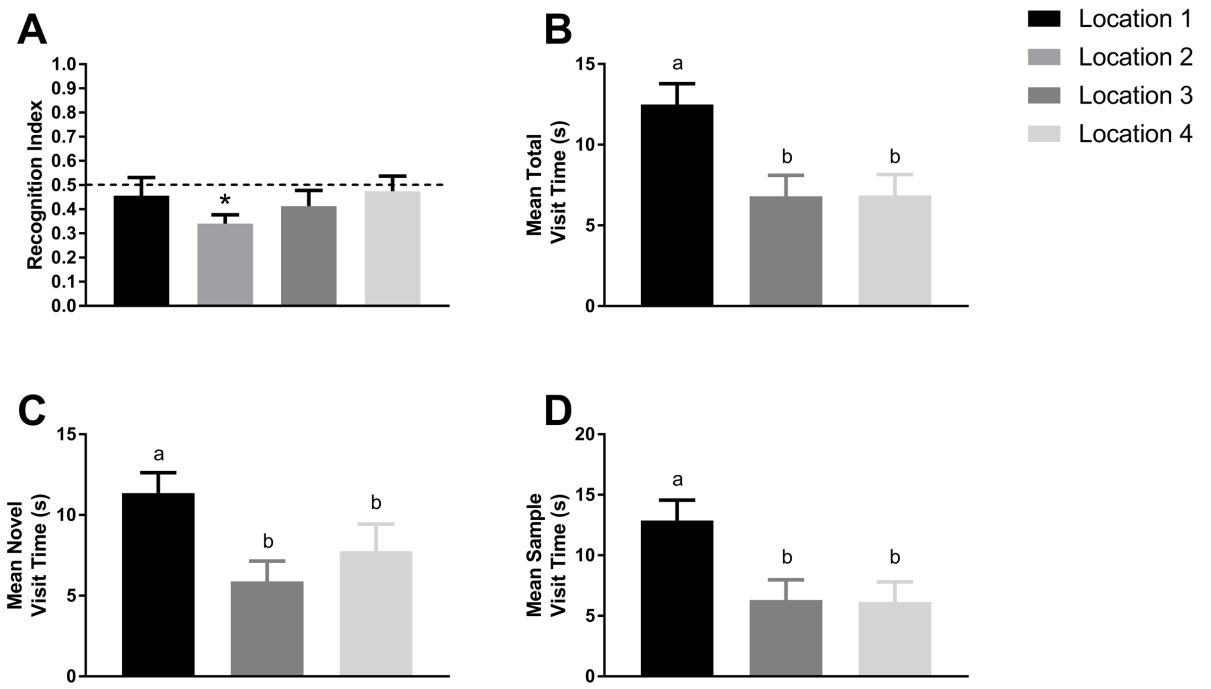

Figure 2.6: Effect of object location on innate preference for the spatial arrangement of objects and exploratory behavior in a one trial control task. **A** Recognition index scores for object locations 1-4. Note location 2 had a recognition index score below 0.50 as determined by one-tailed t-test (marked by asterisk), indicating a preference for the location used as the sample stimulus. This location was thus removed from subsequent analyses. **B-D)** Mean time spent investigating the sample, novel, or both object locations per visit. In all cases, location 1 elicited a greater mean object visit time. These data display the importance of counterbalancing object locations used even after omitting object pairs that elicit innate preferences. All values are least square means ± pooled SEM (individual SEM reported for panel A). Significance between groups (indicated by letter superscript) determined by repeated measures mixed model ANOVA with Tukey adjustment.

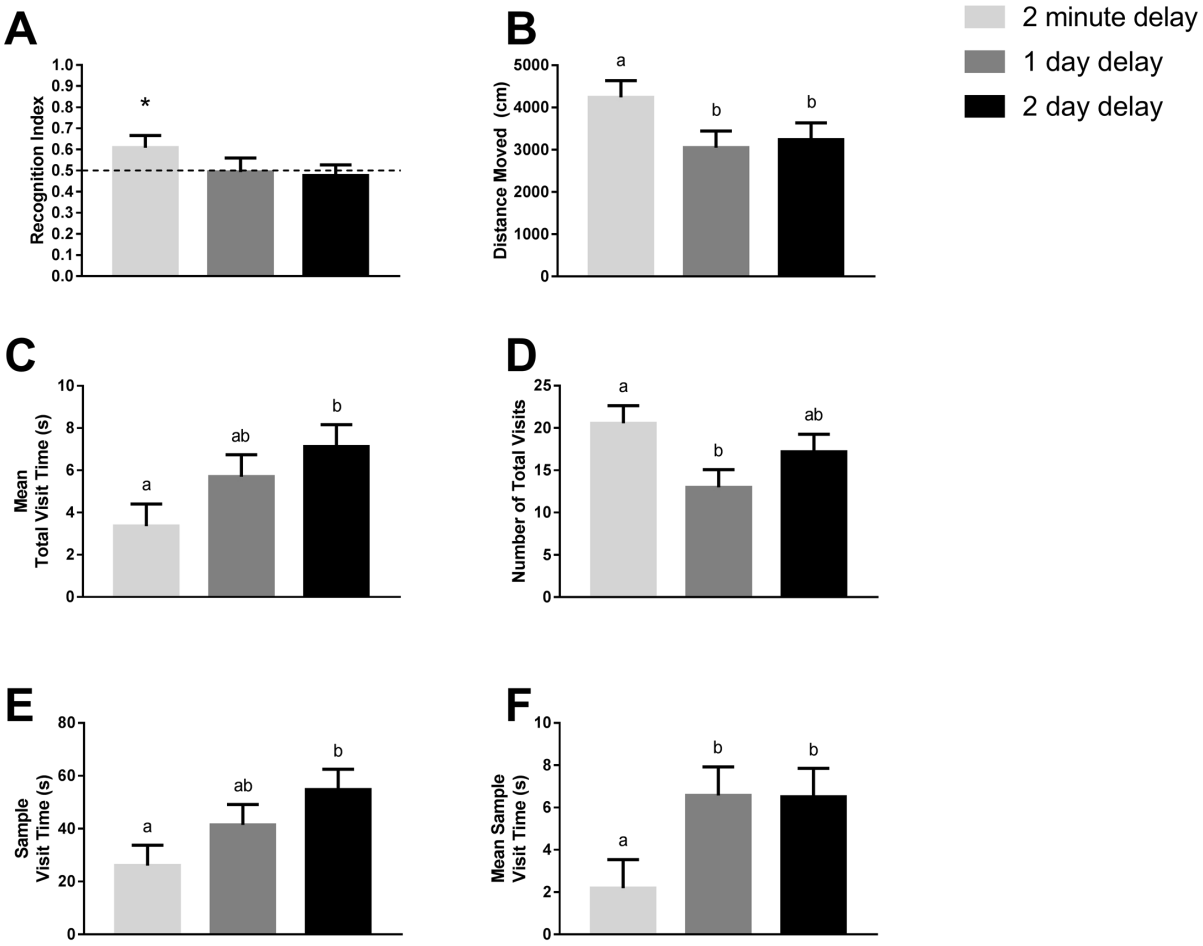

Figure 2.7: Effect of delay on exploratory behavior in pigs subjected to a novel location recognition task at postnatal week 3. **A)** Recognition indices for each delay. Piglets exhibited a recognition index greater than 0.50 for only the 2-minute delay as determined by one tailed t-test (marked by asterisk). There were no differences between groups. **B)** Piglets displayed increased distance moved during the 2-minute delay. **C)** Mean total visit time increased as delay length increased. **D)** Number of total visits to objects was different between groups, but not in a linear fashion. **E)** Total sample object visit time increased as delay length increased. **F)** Mean sample visit time was increased in the 1- and 2-day delay as compared to the 2-minute delay. All values are least square means ± pooled SEM (individual SEM reported for panel A). Significance between groups (indicated by letter superscript) determined by repeated measures mixed model ANOVA with Tukey adjustment.

Chapter 3

Dietary polydextrose and galactooligosaccharide improve recognition memory[1]

3.1 Abstract

Previous studies have shown that dietary prebiotics have the potential to improve memory, alter social behavior, and reduce anxiety-like behaviors in rodents. The present research sought to expand upon such results and describe the effects of feeding prebiotics early in life on cognition and neurochemistry using a translational piglet model. Pigs were provided customized milk replacer containing 2 g/L each of polydextrose (PDX) and galactooligosaccharide (PDX/GOS) or 0 g/L (Control) from postnatal day (PND) 2-33. Beginning on PND 25, pigs were tested on the novel object recognition (NOR), novel location recognition (NLR), and backtest tasks to measure recognition memory and response to restraint stress. At study conclusion pigs were euthanized and intestine, blood, and brain tissues were collected and analyzed. PDX/GOS-fed pigs demonstrated recognition memory on the NOR task ($p < 0.001$) whereas Control pigs did not ($p = 0.184$). Additionally, PDX/GOS-fed pigs visited the novel and sample objects more frequently (all $p < 0.05$) while spending less time per visit exploring the sample object ($p = 0.028$) than Control pigs. Volatile fatty acids (VFAs) were decreased in the ascending colon ($p < 0.012$), whereas butyrate tended to be higher in blood ($p = 0.080$) and lower in the hippocampus ($p = 0.061$) of PDX/GOS fed pigs. PDX/GOS-fed pigs exhibited lower serotonin ($p = 0.016$) in the hippocampus. These findings suggest that early life consumption

[1]Fleming, S.A., Monaikul, S., Patsavas, A.J., Waworuntu, R.V., Berg, B.M., and Dilger, R.N. 2017. Dietary polydextrose and galactooligosaccharide increase exploratory behavior, improve recognition memory, and alter neurochemistry in the young pig, *Nutritional Neuroscience*. 1–14. The copyright owner has provided permission to reprint.

of PDX/GOS supports recognition memory as measured by NOR while modulating the concentrations of VFAs in the colon, blood, and brain, as well as hippocampal serotonin.

3.2 Introduction

It is widely accepted that breast milk is the preferred source of nutrition for infants, however in many situations infants are unable to receive breast milk, and must rely on infant formula as either a partial or whole source of nutrition (Martin et al., 2016). Evidence suggests that the incorporation of prebiotic substrates in infant formula may have beneficial effects on intestinal function (Bertelsen et al., 2016, Vandenplas et al. (2013)). Two commercially available prebiotics that are added to some infant formulas include polydextrose (PDX) and galactooligosaccharide (GOS).

PDX is a non-digestible polysaccharide composed of a highly branched glucose polymer. It is slowly fermented in the large intestine by host microbiota, with approximately 60% excreted in feces (do Carmo et al., 2016). Consumption of PDX in adults has been shown to increase absorption of calcium, magnesium, and iron (Legette et al., 2012, Albarracín et al. (2014), Freitas dos Santos et al. (2009)), improve glucose and lipid metabolism (Tiihonen et al., 2011, Konings et al. (2014)), and increase the presence of beneficial bacteria (Konings et al., 2014, Costabile et al. (2012)). Infant consumption of formula containing both PDX and GOS brought stool consistency and fecal counts of *Bifidobacterium longum* and total *bifidobacteria* counts closer to that of breastfed infants when compared to infants consuming formula without PDX and GOS (Scalabrin et al., 2012). Galactooligosaccharides are found in numerous structural configurations, and are typically composed of galactose monomers linked together by β-glycosidic linkages with a terminal glucopyranosyl residue linked via an α-glycosydic bond.(Playne and Crittenden, 2009) Supplementation of infant formula with GOS supports the selective growth of *bifidobacteria* and *lactobacilli* species (Ben et al., 2008, Macfarlane et al. (2008)), and provision of formula containing GOS has also been shown to reduce intestinal infection (Bruzzese et al., 2009) and incidence of allergy (Arslanoglu et al., 2008) in infants.

In addition to their gastrointestinal effects, emerging evidence suggests some prebiotics may promote brain development and cognition. Rodents fed PDX and GOS for 28–30 days during early postnatal development displayed increased positive social interactions and recognition memory (Waworuntu et al., 2014). Furthermore, evidence for GOS and fructooligosaccharide (FOS) has repeatedly established a role for both prebiotics to alter the molecular expression of learning and memory-related proteins. Oral gavage with 3 g of FOS/kg body weight (BW) or 4 g of GOS/kg BW for 5 weeks has been shown to differentially alter central brain derived neurotrophic factor (BDNF), N-methyl-D-aspartate receptor (NMDAR), and plasma D-serine in adult male rats (Savignac et al., 2013). In a follow-up study, the same dose of GOS (4 g/kg) provided by oral gavage during neonatal development (postnatal days (PND) 3-21) increased BDNF, synaptophysin, and the NMDAR subunit GluN2A at PND 22, which persisted after supplementation ended up to PND 56 in rats (Williams et al., 2016). Galactooligosaccharide supplementation in drinking water

was later shown by the same group to reduce lipopolysaccharide (LPS)-induced anxiety-like behavior and normalize serotonin receptor and cytokine expression in adult male mice (Savignac et al., 2016). In a human trial, GOS supplementation reduced the waking cortisol response and reduced attentional vigilance towards negative stimuli as compared to maltodextrin- or FOS-supplemented controls. Additionally, FOS, chitosan oligosaccharides, and 2 fucosyllactose have also been shown to improve cognition in rodent models (Yen et al., 2017, Jia et al. (2016), Vázquez et al. (2015)).

Provided the growing body of literature demonstrating a beneficial impact of prebiotic supplementation on cognition, the present study was designed to assess the effects of early life prebiotic supplementation (combination of PDX and GOS; PDX/GOS) on cognition using a translational piglet model. The piglet is an optimal pre-clinical model for testing nutritional interventions given their similarity to humans in gastrointestinal physiology (Wang and Donovan, 2015, Odle et al. (2014)), nutritional requirements (Mudd and Dilger, 2017), and brain development (Lind et al., 2007, Sauleau et al. (2009), Kornum and Knudsen (2011)). We supplemented pigs from PND 2-33 with polydextrose and galactooligosaccharide (PDX/GOS) and measured their performance on several behavioral tasks. The novel object recognition (NOR) test and novel location recognition (NLR) test were chosen to assess object and spatial recognition memory, and the backtest was chosen to assess response to restraint stress. To understand possible gut-brain-axis mechanisms we measured volatile fatty acids (VFAs) in the large intestine, blood, and brain, plasma non-esterified fatty acids (NEFA), and investigated expression of memory-related proteins and catecholamines in the brain.

3.3 Methods

3.3.1 Animals and housing

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. Beginning at PND 2, 24 naturally farrowed intact male pigs were artificially reared to PND 33. The trial was completed in two replicates (12 pigs per replicate), with 12 pigs per treatment, selected from 6 litters, to control for genetics and initial body weight (BW). One pig (Control diet) was removed from study due to insufficient weight gain and failure to thrive. Pigs were housed in cages (L $\times$ W $\times$ H of 87.6 $\times$ 88.9 $\times$ 50.8 cm) constructed of clear acrylic and stainless steel with vinyl-coated metal flooring (Tenderfoot™) that allowed pigs to see, hear, smell, but not touch, neighboring pigs. Ambient room temperature was maintained between 27°C and 29°C and a 12 h light/dark cycle was maintained with light from 0800 to 2000h. Euthanasia procedures were conducted as previously described (Mudd et al., 2016a).

3.3.2 Dietary groups

Pigs (n = 12 per diet) were provided a custom milk replacer containing 2 g/L each of PDX (Danisco, Terre Haute, IN, USA) and GOS (FrieslandCampina, Zwolle, Netherlands) (PDX/GOS) or 0 g/L (Control) from PND 2-33. Milk replacer was reconstituted at 200 g of dry powder per 800 g of water. Pigs were fed at 285 and 325 mL of reconstituted milk replacer per kg BW beginning on PND 2 and 8, respectively. Body weight was recorded daily to determine the volume of milk to be dispensed to individual animals throughout the day. Meals were administered 10 times a day approximately every 2 hours from 1000 h on one day to 0400 h the subsequent day and each diet was reconstituted fresh before the start of each feeding cycle.

3.3.3 Behavioral testing

Pigs were tested on the NOR and NLR tasks to assess perirhinal- and hippocampal-dependent memory. Testing consisted of a habituation phase, a sample phase, and a test phase. During the habituation phase, each pig was placed in an empty testing arena for 10 min each day for two days leading up to the sample phase. In the sample phase, the pig was placed in the arena containing two identical objects and given 5 min for exploration. After a delay of 45- or 24-h the pig was returned to the arena for the test phase of the NOR and NLR tasks, respectively. During the test phase, the pig was placed in the arena containing one object from the sample phase as well as either a novel object for the NOR task or a sample object moved to a novel location for the NLR task and allowed to explore for 5 min. Between trials, objects were removed, immersed in hot water with detergent, and rubbed with a towel to mitigate odor, and the arena was sprayed with water to remove urine and feces. Objects chosen had a range of characteristics (i.e. color, texture, shape, and size), however the novel and sample objects only differed in shape and size. Only objects previously shown to elicit a null preference were used for testing (Fleming and Dilger, 2017). Task order was counterbalanced between replicates. Habituation trials began at PND 25, and testing on either the NOR or NLR tasks began at PND 27. The recognition index, the proportion of time spent with the novel object/location compared to total exploration of both objects, was used to measure recognition memory. A recognition index significantly above 0.50 demonstrates a novelty preference and thus recognition memory.

3.3.3.1 Backtest

The backtest was used as a measure of response to restraint stress. On PND25 and 32, pigs were restrained on a V-shaped board by a single experimenter for 60 seconds. A ceiling-mounted camera was used to record each subject's performance. Number of struggling bouts, duration of struggling bouts, mean duration of struggle bouts, and latency to first struggle bout were recorded and analyzed manually by a trained experimenter blind to dietary group.

3.3.4 Sample collection and assessment

Pigs were humanely euthanized at PND 33. Samples collected and stored for subsequent analyses included brain tissue (hippocampus, striatum, and medial prefrontal cortex), blood, feces, and luminal contents from the cecum and large intestine. All samples, except cecal and ascending colon contents, were snap frozen in liquid nitrogen and stored at −80°C. Frozen brain tissue was then mechanically homogenized in liquid nitrogen using a Freezer/Mill (SPEX, USA) and the powdered samples stored at −80°C. The small intestine of each pig was examined to permit quantification of macrostructural characteristics (i.e. length and empty weight of each segment).

3.3.4.1 Analysis of VFAs and NEFA

Fresh cecal and ascending colon luminal content samples (100 mg each) were weighed and immediately preserved for quantification of VFAs using acidification with an equal volume of HCl. Blood (1 ml), brain tissue (100 mg), and feces (100 mg) were thawed and acidified using 6.25% m-Phosphoric acid, sonicated, and stored overnight at −20°C. Blood and brain samples were thawed and centrifuged for ten minutes at 16,500 × g, and supernatant was extracted and reconstituted with equal volume of ethyl acetate and vortexed for two minutes. Samples were then centrifuged again for ten minutes at 16,500 × g and supernatant was used for analysis via gas chromatography. In brief, VFA concentrations for cecal, ascending colon, and fecal contents were assessed as previously described (Smiricky-Tjardes et al., 2003), whereas blood and brain samples were assessed as described by García-Villalbe et al. (García-Villalba et al., 2012). Acetic, n-butyric, propionic, isovaleric, valeric, and isobutyric acid solutions (Sigma-Aldrich, USA) were used as standards. Freshly collected blood was centrifuged at 1,160 × g and plasma was extracted and stored at −80°C until further processing. Samples were analyzed for NEFA concentrations as described previously (Wako Pure Chemical Industries, 2012) using the Beckman Coulter Olympus AU680 analyzer (Beckman Coulter, USA).

3.3.4.2 Brain catecholamine analysis

Absolute quantification of the following catecholamines were conducted in duplicate using validated liquid chromatography methods: dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine, epinephrine, 5-hydroxytryptamine (serotonin) and 5-hydroxyindoleacetic acid (HIAA). In brief, high-performance liquid chromatography (HPLC) methods based on the Dionex Application Note 228 were used for rapid and sensitive separation and quantification using electrochemical detection (Dionex, 2009). Method B of this procedure was employed to simultaneously quantify all the aforementioned biochemicals in brain samples, and extraction procedures were optimized for extraction of catecholamines from pig brain tissue samples.

3.3.5 Western blotting

3.3.5.1 Sample preparation

Hippocampus, medial prefrontal cortex, whole mouse brain (used as a positive control), and liver (used as a negative control) tissue samples were reconstituted at a concentration of 100 mg/ml in 1 ml 1X lysis buffer containing 1X phenylmethylsulfonyl fluoride. Samples were homogenized via mechanical bead separation using the TissueLyser II (Qiagen, Germany) at 30 hz for 60 seconds. This process was repeated until samples were homogenized and no observable clusters of tissue remained. Samples were lysed via sonication twice at 15-second intervals, with the sonicator (Model 120 Sonic Dismembrator, Fisher Scientific, USA) set at 100%. Samples were then centrifuged (5430R, Eppendorf, Germany) at 14,000 × g at 4°C for 10 min, and the resulting supernatant was aliquoted and stored at −20°C until blotted.

3.3.5.2 Protein concentration and quantification

A bicinchoninic acid (BCA) protein assay (Pierce BCA Protein Assay Kit, #23227, ThermoFisher Scientific, USA) was performed on the supernatant to quantify protein concentrations according to the manufacturer's specifications, with quantification performed using a microplate reader (Biotek Synergy HT, Biotek Instruments, USA).

3.3.5.3 Western blot analysis

All steps were performed at room temperature. Protein samples (20 μg/well) were separated on NOVEX 4–12% bis-tris gels (NW04125BOX, ThermoFisher Scientific, USA) and transferred using the iBlot Gel Transfer Device with nitrocellulose (0.2 μm pore size) iBlot Gel Transfer Stacks per instructions provided with the iBlot system (ThermoFisher Scientific, USA). Immediately after transfer, membranes were blocked in 0.1% Ponceau containing 5% acetic acid (P7170, Sigma-Aldrich, USA) for 5 min, preceding two 3-min washes in distilled water. Membranes, now stained for total protein, were imaged using the Bio-Rad ChemiDoc MP System (Bio-Rad Laboratories, USA) and Ponceau dye was removed by washing in 0.1M NaOH until clear. Blocking, antibody incubation, and wash steps were performed using procedures as described by the SNAP i.d. 2.0 Protein Detection System (SNAP2MINI, EMD Millipore, Germany). A 5% solution of BSA (A2153, Sigma-Aldrich, USA) diluted with 1X TBS + T (prepared by the addition of 10% Tween 20 (161-0781, BioRad Laboratories, USA) to TBS) was used as the blocking buffer. Proteins of interest included: synaptophysin (1:40,000 dilution, ab32127, Abcam, UK), post-synaptic density 95 protein (PSD-95, 1:1000 dilution, 56477, QED Bioscience, USA), and cAMP response element-binding protein (CREB, 1:1,000 dilution, 9104, Cell Signaling Technology, USA). A goat anti-mouse or anti-rabbit antibody conjugated to alkaline phosphatase (1:3,000 dilution, 170-5010/ 1, Bio-Rad Laboratories, USA) was used for the secondary antibody.

Protein expression was imaged using the Bio-Rad ChemiDoc MP System (Bio-Rad Laboratories, USA)

after chemiluminescent detection using the Immun-Star AP Chemiluminescent kit (64056423, Bio-Rad Laboratories, USA). Bands were analyzed using Imagelab 5.2.1 software (Bio-rad, USA). To control for the amount of protein loaded, all lanes were normalized by using the total protein stain as a standardizing marker. To control for the use of multiple gels, band intensity was measured as a relative value by dividing the volume intensity of each band by a reference band of the same molecular weight from mouse brain tissue. Bands were chosen by selecting those closest to the molecular weight predicted by the vendor: synaptophysin, 34–38 kDa; PSD-95, 95–100 kDa; CREB, 43 kDa.

3.3.6 Statistical analysis

All researchers involved with conducting the study, including data acquisition and analysis phases, remained blind to dietary treatment identity until final statistical analyses had been completed. All data generated as part of this study were subjected to analyses of variance (ANOVA) using the MIXED procedure of SAS Enterprise Guide 5.1 (SAS Inst. Inc., Cary, NC, USA). All statistical models included replicate as a random effect and the level of significance was set at $p < 0.05$, with trending significance set to $0.05 \leq p < 0.10$. Depending on the outcome, one of two statistical models was used: 1) data collected at a single time-point (e.g. biochemical analyses on brain tissue, VFA concentrations, etc.) were analyzed by a 1-way ANOVA, and 2) data collected from the same animal on more than one occasion (e.g. daily BW, behavioral outcomes, etc.) were analyzed as a 2-way repeated measures ANOVA. For some behavioral outcomes (e.g. comparing the NOR recognition index to a null value), a one-sample t-test was conducted. For all analyses, data points were removed from analyses if they were greater than three studentized residuals from the mean. For some variables, transformations were required when data violated the homogeneity of variance assumption. In cases where transformations did not satisfy the homogeneity of variance, an alternative test was conducted. A correlation matrix was computed and visualized using the corrplot function (Wei and Simko, 2016) in R (v. 3.2.2, R Core Team, Austria); only significant correlations ($p < 0.05$) are shown. All supplemental tables and figures can be found at the journal article (Fleming et al., 2017)

3.4 Results

3.4.1 Growth performance

Over the course of study, a main effect of age ($p < 0.001$) and diet ($p < 0.001$) were observed for daily BW, as BW increased with age and Control pigs exhibited overall heavier BW. While no interactive effect was observed, post-hoc comparisons revealed that Control pigs were significantly heavier than PDX/GOS-fed pigs only on PND 32 ($p = 0.035$, Figure 3.1). No dietary differences were observed for lengths or weights of small intestine segments (all $p \geq 0.119$, Supplemental Table 1).

3.4.2 VFAs and plasma NEFA

All VFAs are expressed as a proportion of sample dry weight (for cecal, ascending colon, and fecal samples). No differences in cecal or fecal concentrations of VFA were observed (all $p \geq 0.197$). However, higher concentrations of all VFA quantified in ascending colon luminal content samples were observed for Control pigs as compared with pigs fed PDX/GOS (all $p \leq 0.012$). Both acetate and butyrate were detected in blood and brain samples, with the concentration of butyrate in blood trending higher in pigs fed PDX/GOS ($p = 0.080$), whereas hippocampal butyrate was lower in pigs fed PDX/GOS ($p = 0.061$, Figure 3.2). There were no differences in VFA observed in the striatum (all $p \geq 0.258$). Molar ratios of all VFA in all samples remained unchanged by diet (all $p \geq 0.142$), however percent acetate trended higher and percent isobutyrate trended lower in feces of pigs fed PDX/GOS (both $p \leq 0.099$). Luminal contents of the PDX/GOS-fed pigs were higher in percent dry matter in the ascending colon but lower in feces compared to Control pigs (all $p \leq 0.035$, Supplemental Table 2). Plasma NEFA trended lower in pigs fed PDX/GOS ($p = 0.090$, Figure 3.2).

3.4.3 Behavioral outcomes

Pigs fed PDX/GOS displayed a novelty preference (an indicator of recognition memory) as evidenced by a recognition index significantly above 0.50 on the NOR task ($p < 0.001$), but Control pigs did not ($p = 0.184$, Figure 3.3). Neither group showed a novelty preference in the NLR task (all $p \geq 0.320$, Supplemental Table 3). During the test trial of the NOR task, pigs fed PDX/GOS exhibited a greater recognition index ($p = 0.045$), number of novel object visits ($p = 0.039$), number of sample object visits ($p = 0.049$), and shorter mean sample object visit time ($p = 0.028$) compared with Control pigs (Figure 3.3). For a full analysis of all behaviors measured including trending effects and behavior during habituation or sample trials, see supplemental material (Supplemental Table 4). There was no effect of diet for any measures on the backtest (all $p \geq 0.561$), however pigs spent less time struggling when tested on PND 32 as compared to PND 25 ($p = 0.002$, Table 3.1).

3.4.4 Brain catecholamine and western blot analyses

Pigs provided PDX/GOS exhibited lower ($p = 0.016$) concentrations of serotonin in the hippocampus compared with Controls, and a trend for higher HVA and lower serotonin in the striatum (all $p \leq 0.062$, Table 3.2). No effect of diet was observed for expression of CREB, PSD-95, or synaptophysin in either the hippocampus or medial prefrontal cortex (all $p \geq 0.336$, Table 3.3, Figure 3.4).

3.5 Discussion

The objective of this study was to assess the effects of early life prebiotic supplementation on cognition in a piglet model of infancy. We supplemented pigs from PND 2 through 33 with PDX/GOS and measured their performance on several behavioral paradigms, quantified concentrations of VFA in the large intestine, blood, and brain, and investigated expression of memory-related proteins and catecholamines in the brain. Evidence from this study revealed dietary PDX/GOS reduced BW, improved performance on the NOR task, altered concentrations of VFA in the ascending colon, blood, and brain, and reduced the concentration of serotonin in the hippocampus. Neither dietary group was able to complete the NLR task, nor did supplementation with PDX/GOS alter performance on the backtest or change expression of CREB, PSD-95, or synaptophysin.

Pigs fed PDX/GOS exhibited reduced BW over the course of the study, however the rate of growth and ultimate BW were well within the normal range for artificially reared pigs (Berding et al., 2016). It may be likely that the reduction in BW is related to modulation of satiety, and thus food intake, which may be surmised from a previous study wherein Wistar rats fed GOS exhibited reduced food intake and increased expression of the satiety-related peptides PYY and proglucagon (Overduin et al., 2013).

However, several studies in human infants and rodents have shown that feeding with the combination of PDX and GOS or GOS alone produced negligible differences in BW (Waworuntu et al., 2014, Savignac et al. (2013), Ashley et al. (2012)). In our study, milk replacer was provisioned in a feeding paradigm designed to mimic ingestion patterns for sow-reared pigs, and it should be noted that we were not able to accurately quantify food intake. Thus, we cannot definitively conclude that the reduction in BW gain was due to reduced food intake.

We observed reduced VFA concentrations in the ascending colon and no difference in either the cecum or feces of pigs fed PDX/GOS compared with Control animals. In general, evidence regarding changes in colonic VFA concentrations after ingesting dietary prebiotic substances is mixed. Human and animal studies have shown that PDX either reduces colonic VFA concentrations (Peuranen et al., 2004, Fava et al. (2007)) or has no effect on fecal VFA concentrations (Costabile et al., 2012, Hengst et al. (2009)). *In vitro* evidence suggests that addition of PDX to colonic simulators increases production of VFA (Mäkeläinen et al., 2007, Probert et al. (2004)), but production is generally lower than shorter chain prebiotics (Hernot et al., 2009, Mäkeläinen et al. (2010), Li et al. (2012)). Similarly, in infants, GOS has been shown to increase fecal VFA concentrations (Ben et al., 2004), lower the proportion of fecal butyrate (Sierra et al., 2015), or have no effect on fecal total VFA concentrations (Sierra et al., 2015, Bakker-Zierikzee2005a). However, *in vitro* models have been used to demonstrate that GOS is rapidly fermentable (Boler et al., 2013), increases acetate and butyrate production (Grimaldi et al., 2017), and simultaneously increases acetate while lowering concentrations of all other VFA (Musilova et al., 2015). Collectively, these data suggest there is no clear conclusion as to how PDX or GOS affect colonic or fecal VFA concentrations *in vivo*.

3.5.1 Prebiotics and behavior

Overall, our study demonstrated that PDX/GOS elicited a notable influence on recognition memory and exploratory behavior. We chose to use the NOR task in this study as it is a novelty-preference-based task, which is a widely used paradigm for assessment of recognition memory in infants (Rose et al., 2004), thus increasing its translatability to pediatric nutrition as compared with maze-based operant tasks. In addition to improving recognition memory, we observed a greater number of visits to both the sample and novel objects and reduced time spent per visit to the sample object in pigs fed PDX/GOS. Such changes in behavior are similar to those found in a previous study from our laboratory investigating the effect of age on performance in the NOR task; 4-week-old pigs spent more time exploring both novel and sample objects compared with the same pigs at 3 weeks of age (Fleming and Dilger, 2017). This suggests that the increase in exploratory behavior of pigs fed PDX/GOS was an improvement in performance, rather than abnormal behavior. Neither group was able to complete the NLR task. We chose to include the NLR task as previous work demonstrated the NLR task is more difficult for pigs to perform at 4 weeks of age than the NOR task (Fleming and Dilger, 2017). Had the NOR task been too simple for both groups, the NLR task was included to provide a more challenging test of recognition memory, however it proved too difficult for either group in the current study. Our results replicate recent findings that weanling Long-Evans rats fed PDX/GOS displayed increased recognition memory after a 48-h delay when compared with control rats (Waworuntu et al., 2014). Furthermore, supplementation with either FOS or chitosan oligosaccharides reduced cognitive dysfunction in rodent models of Alzheimer's disease (Yen et al., 2017, Jia et al. (2016)), and providing the human milk oligosaccharide 2 -fucosyllactose to adult mice increased hippocampal long-term potentiation, improved memory consolidation, and enhanced spatial and associative learning (Vázquez et al., 2015, Vazquez et al. (2016)).

It has been hypothesized that microbial metabolites may serve to influence behavior (Sampson and Mazmanian, 2015), yet it is unclear how they work together, if at all, to facilitate improvements in memory. The only relationship found between recognition index and VFA was negative with isobutyrate in the ascending colon. As there were no relationships between blood or brain VFA and recognition index, it is unlikely that VFA act via a humoral route on the brain to improve memory. Instead, we found numerous relationships between VFA and exploratory behavior in the NOR test trial (Figure 3.5). Interestingly, there were more relationships found between exploratory behavior and VFA in the ascending colon (i.e. the region most affected by feeding PDS/GOS) than in other tissues. This finding suggests there may exist an unknown mediator between VFA and exploratory behavior that acts preferentially in the ascending colon. This mediator may involve the vagus nerve itself or a factor that is vagally mediated. Indeed, a recent study has shown that long-term potentiation in response to dietary 2-fucosyllactose requires vagal communication (Vazquez et al., 2016). Whether this communication is due to vagal sensing of VFA or other neuroactive bacterial metabolites such as catecholamines and growth factors is not understood. Assuming

that communication is indeed vagally mediated, it was surprising that the only VFA to correlate with the recognition index was ascending colon isobutyrate. There is mounting evidence that VFA may play a role in the gut-brain-axis as modulators of blood–brain barrier integrity (Braniste et al., 2014) or as HDAC inhibitors (Bourassa et al., 2016), and our evidence suggest that endogenous VFAs are additionally related to many aspects of exploratory behavior, but minimally related to memory.

To date, studies investigating modulation of the microbiota via prebiotics, probiotics, or using germ-free models have consistently reported effects on anxiety, depression, or social behavior in both humans (Messaoudi et al., 2011b, Steenbergen et al. (2015b), Tillisch et al. (2013)) and rodents (Clarke et al., 2013, Heijtz et al. (2011), Neufeld et al. (2011), Selkrig et al. (2014), Bravo et al. (2011), Savignac et al. (2014), Desbonnet et al. (2014)). Although PDX/GOS fed pigs displayed distinctly different behavior for learning and memory-related outcomes, they did not show altered behavior for any measure on the backtest. Previous research has categorized pigs according to coping style as 'high-' or 'low-resisting' depending on whether they exhibit many or few escape behaviors during the backtest (Bolhuis, 2004). These phenotypes appear stable across time and are related to the hypothalamic–pituitary–adrenal axis function, aggression, and behavioral response to dopaminergic agonists (Bolhuis, 2004). In support of these relationships, we found that striatal HVA, hippocampal dopamine, and PSD-95 were all negatively correlated with mean length of struggle bout (Figure 3.5). Additionally, we found a negative relationship between fecal butyrate and time spent struggling and mean length of struggle bout (Figure 3.5), suggesting those with less fecal butyrate tend to attempt escaping for longer periods of time, similar to a previous report demonstrating a link between anxiety-like behaviors and cecal VFA concentrations (Hanstock et al., 2004). We did not measure physiological stress or aggressive behavior in this study, but if the relationship between coping styles, aggression, and stress are substantial, it may be worthwhile to investigate the involvement of fermentation products such as butyrate.

3.5.2 Brain catecholamines and protein expression

Several reports have shown increases in proteins related to synaptic plasticity and cognition in response to ingestion of dietary prebiotics. Neonatal rats fed diets containing GOS from PND 3-21 displayed increased expression of BDNF, synaptophysin, and the NMDAR receptor subunit GluN2A that persisted to PND 56, well after supplementation had ceased (Williams et al., 2016). Supplementation with GOS has also been shown to increase expression of BDNF and the NMDAR subunits NR2A and NR1A in the hippocampus and frontal cortex (Savignac et al., 2013), however neither of these studies investigated cognitive effects. Vazquez et al. (Vázquez et al., 2015) demonstrated an improvement in cognition coincident with increases in PSD-95 in the hippocampus and frontal cortex, CamKII in the hippocampus, and BDNF in the hippocampus and striatum in adult male rats fed 2 fucosyllactose, an oligosaccharide with prebiotic potential found in mammalian milk. Other reports have shown that chitosan oligosaccharides and FOS can improve cognition

and reduce markers of Alzheimer's pathology (Yen et al., 2017, Jia et al. (2016)) and GOS may also normalize aspects of the inflammatory response to LPS (Savignac et al., 2016).

Given the consistent effect of prebiotics on synaptic-related proteins, we chose to investigate the expression of PSD-95, CREB, and synaptophysin, proteins known to be critical for learning, memory, and synaptic transmission (Wang and Peng, 2016, Gordon and Cousin (2014)). We did not observe effects on brain expression of these proteins, possibly due to our emphasis on early life PDX/GOS supplementation in the overall timeline of brain development in the pig. Nearly all previous studies involved supplementation of rodents with prebiotics for a greater relative time span (Savignac et al., 2013, Williams et al. (2016), Vázquez et al. (2015)), or used prebiotic supplementation in concert with a disease or immune challenge (Savignac et al., 2016, Yen et al. (2017), Jia et al. (2016)). Prebiotics may confer neuroprotective effects in response to an insult, however such effects were not studied here. In this cross-species comparison, the same time span in a rodent encompasses a greater portion of development than in a pig, and therefore pigs in our study may have exhibited altered protein expression had we continued the experiment beyond 4-weeks of life. Gastrointestinal development of the pig undergoes rapid growth at birth and weaning but continues to mature up to 12- weeks of age (Sangild, 2006). Furthermore, we acknowledge that significant changes in brain growth and maturation continue through approximately 24 weeks of age in the domestic pig (Conrad et al., 2012a).

Whereas brain expression of key proteins involved in learning and memory were largely unchanged, pigs fed PDX/GOS displayed reduced serotonin in the hippocampus, and while not statistically significant, a similar magnitude of reduced serotonin was also observed in the striatum. Reduced serotonin in these regions may be related to central regulation of stress behavior. In response to inescapable stress, the receptor 5-HT1AR plays a protective role by inhibiting serotonergic neurons in the dorsal raphe nucleus (Rozeske et al., 2011). A recent study showed that a stress induced reduction in 5HT1AR was attenuated after rats were fed diets containing PDX, GOS, and lactoferrin (Mika et al., 2017). Although we saw no differences due to diet in the backtest, our study employed a mild stressor in comparison to Mika et al., (Mika et al., 2017) whose study employed a model of inescapable stress and learned helplessness.

The majority of serotonin in the body is produced by enterochromaffin cells in the gut (Gershon and Tack, 2007), however as serotonin cannot cross the blood brain barrier, it is unlikely that alteration of peripheral serotonin production reduced serotonin in the hippocampus and striatum via a humoral route. It may be possible that availability or metabolism of tryptophan (Trp) was reduced or shunted toward greater kynurenine synthesis than serotonin. We measured plasma NEFA as they compete with Trp for binding to albumin, thus affecting the pool of Trp available to cross the blood brain barrier (Ruddick et al., 2006). Here, pigs fed PDX/GOS displayed a moderate decrease in plasma NEFA, which may have led to an increase in transport of Trp into the brain, but we are unable to provide direct confirmatory evidence as brain Trp concentrations were not quantified in our study.

There is little information on the relationship between prebiotics and Trp metabolism, however several studies have established that modulation of the microbiome via germ-free conditions results in alterations to circulating Trp and serotonin. Peripherally, germ-free mice display reduced serotonin in serum (Wikoff et al., 2009, Yano et al. (2015)), plasma (Yano et al., 2015), colonic contents (Yano et al., 2015), and feces (Yano et al., 2015), increased Trp in serum and plasma (Clarke et al., 2013, Wikoff et al. (2009)), and a reduced plasma kynurenine:tryptophan ratio (Clarke et al., 2013). Centrally, germ-free mice display increased serotonin and its metabolite 5-HIAA in the hippocampus (Clarke et al., 2013), as well as elevated turnover of noradrenaline, dopamine, and serotonin in the striatum (Heijtz et al., 2011). A study in rats showed the opposite trend of germ-free mice, as germ-free rats had reduced serotonin in the hippocampus when compared to specific-pathogen-free controls (Crumeyrolle-Arias et al., 2014). While there are mixed results between rodent species, it is clear that the gut microbiome is heavily involved in regulating both peripheral and central Trp and serotonin metabolism.

Regardless of the root cause, hippocampal serotonin appeared to be related to several measures of exploratory behavior in pigs, but surprisingly, not the NOR recognition index. For example, hippocampal serotonin correlated negatively with visits to the sample object, a characteristic similar to that of pigs fed PDX/GOS, who exhibited less hippocampal serotonin and visited the sample object more frequently than control pigs. Although pigs fed PDX/GOS displayed increased recognition memory and decreased hippocampal serotonin, the two findings did not correlate. Therefore, it is unlikely that the reduction in hippocampal serotonin due to PDX/GOS ingestion detrimentally affected memory, but it appears to be related to several aspects of exploratory behavior.

The general sparsity of identified relationships between the recognition index and VFA, catecholamines, or synaptic proteins raises an important question. What is the mechanism of action between prebiotic supplementation and the observed increase in recognition memory? From our data in the young pig, the strongest connections to recognition index are tenuous between cecal isobutyrate and striatal serotonin. Coincidentally, a negative correlation was identified between striatal serotonin and recognition index, and pigs fed PDX/GOS exhibited higher recognition indices and lower striatal serotonin than controls. Striatal serotonin was also correlated with fecal acetate, propionate, isovalerate, and striatal acetate (Supplemental Figure 1). While this is not direct, causative evidence that decreasing the concentration of striatal serotonin increases recognition index via a mediating action involving VFA, the influence of compartmentalized VFA concentrations on cognition warrants further investigation.

3.6 Conclusions

PDX/GOS in early life had a demonstrable effect on behavior and physiology. Results generated in this study are consistent with previous findings in terms of BW gain and fermentative end-product concentrations, with some notable differences in brain related outcomes. Pigs fed diet containing PDX/GOS exhibited improved

performance on the NOR, but not the NLR test. While ingesting PDX/GOS had no impact on anxiety-like measures in the pig, more refined behavioral analyses are required to confirm this finding. There was no effect of prebiotic consumption on memory-related proteins in the brain, however, reduced serotonin concentrations were detected in the hippocampus and striatum. Correlation analyses revealed relatively few relationships between recognition memory and concentrations of VFA, suggesting prebiotics may improve memory via other non-humoral mechanisms. Future studies aimed at quantifying the underlying mechanism(s) including vagal communication would prove instrumental to understanding how prebiotic ingestion influences cognitive development.

3.7 Supplemental data

Supplemental data for this study can be accessed through the journal *Nutritional Neuroscience* at https://doi.org/10.1080/1028415X.2017.1415280

3.8 Tables

Table 3.1: Effect of diet and age on behavioral response to restraint stress[*†]

	PND 25		PND 32			*p*-value		
Measure[‡§]	Control	PDX/GOS	Control	PDX/GOS	Pooled SEM	Diet	PND	Diet*PND
Total duration of struggling bouts, sec	15.03	14.44	10.61	9.30	1.851	0.561	0.002	0.807
Number of struggling bouts	3.68	4.83	3.49	3.17	0.890	0.557	0.194	0.295
Mean length of struggle bout, sec	4.70	4.16	3.81	3.77	0.923	0.698	0.324	0.704

[*] Abbreviations: PND, postnatal day; SEM, standard error of the mean.
[†] *p*-value from mixed model ANOVA with replicate of pigs as a random effect.
[‡] All pigs exhibited a latency to first struggle bout of 0 seconds.
[§] For all measures: Control, n= 11, PDX/GOS, n= 12.

Table 3.2: Effect of diet on catecholamine concentrations in the brain.[*†]

	Control		PDX/GOS			
Measure[‡]	n	Mean	n	Mean	Pooled SEM	p-value
Hippocampus						
Dopamine	10	0.12	11	0.13	0.035	0.849
DOPAC	9	0.07	9	0.07	0.013	0.953
HVA	-	BDL	-	BDL	-	-
Norepinephrine	11	2.24	12	2.23	0.143	0.943
Epinephrine	11	18.21	12	17.67	6.100	0.862
Serotonin	11	0.22	12	0.16	0.024	0.016
HIAA	11	0.23	12	0.23	0.097	0.988
Medial prefrontal cortex						
Dopamine	10	0.16	12	0.17	0.027	0.641
DOPAC	10	0.16	11	0.16	0.030	0.923
HVA	-	BDL	-	BDL	-	-
Norepinephrine	11	4.76	12	4.17	1.108	0.706
Epinephrine	11	18.92	12	18.88	2.646	0.993
Serotonin	11	0.18	12	0.14	0.016	0.119
HIAA	-	BDL	-	BDL	-	-
Striatum						
Dopamine	10	5.86	12	6.25	0.601	0.261
DOPAC	10	1.10	12	1.17	0.144	0.331
HVA	10	3.77	12	4.31	0.218	0.062
Norepinephrine	11	4.28	12	3.84	0.505	0.538
Epinephrine	11	19.41	12	20.81	3.804	0.677
Serotonin	11	0.21	12	0.17	0.011	0.055
HIAA	11	0.12	12	0.12	0.014	0.937

[*] Abbreviations: n, sample size; SEM, standard error of the mean; BDL, below detectable limit; DOPAC, 3,4- dihydroxyphenylacetic acid; HVA, homovanillic acid; HIAA, 5- hydroxyindoleacetic acid

[†] Data analyzed by mixed model ANOVA with replicate of pigs as a random effect.

[‡] Units are ng/mg wet tissue.

Table 3.3: Effect of diet on catecholamine concentrations in the brain.[*†]

	Control		PDX/GOS			
Measure[‡]	n	Mean	n	Mean	Pooled SEM	p-value
Hippocampus						
CREB	10	0.61	11	0.7	0.072	0.336
PSD-95	10	0.34	11	0.29	0.075	0.409
Synaptophysin	10	0.53	11	0.54	0.176	0.847
Medial prefrontal cortex						
CREB	8	0.27	9	0.28	0.043	0.758
PSD-95	8	0.23	7	0.22	0.066	0.867
Synaptophysin	7	0.57	8	0.7	0.108	0.408

[*] Abbreviations: n, sample size; SEM, standard error of the mean; CREB, cAMP response element-binding protein; PSD-95, post-synaptic density protein-95.

[†] Data analyzed by mixed model ANOVA with replicate of pigs as a random effect.

[‡] Units are arbitrary values.

3.9 Figures

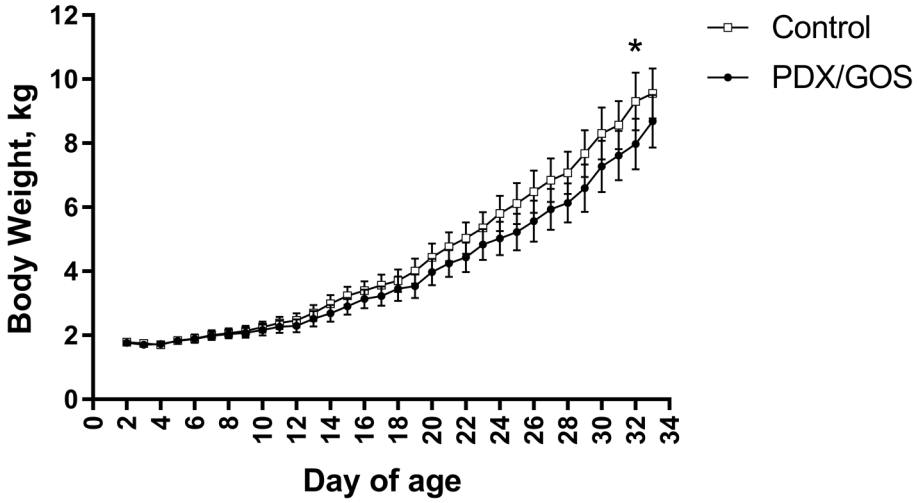

Figure 3.1: BW increased with age ($p < 0.001$) and over the entire feeding study, Control pigs exhibited heavier BWs (main effect of diet; $p < 0.001$). No interactive effect was noted, however, post-hoc comparisons revealed that Control pigs were significantly heavier than PDX/GOS-fed pigs only on PND 32 (* $p = 0.035$).

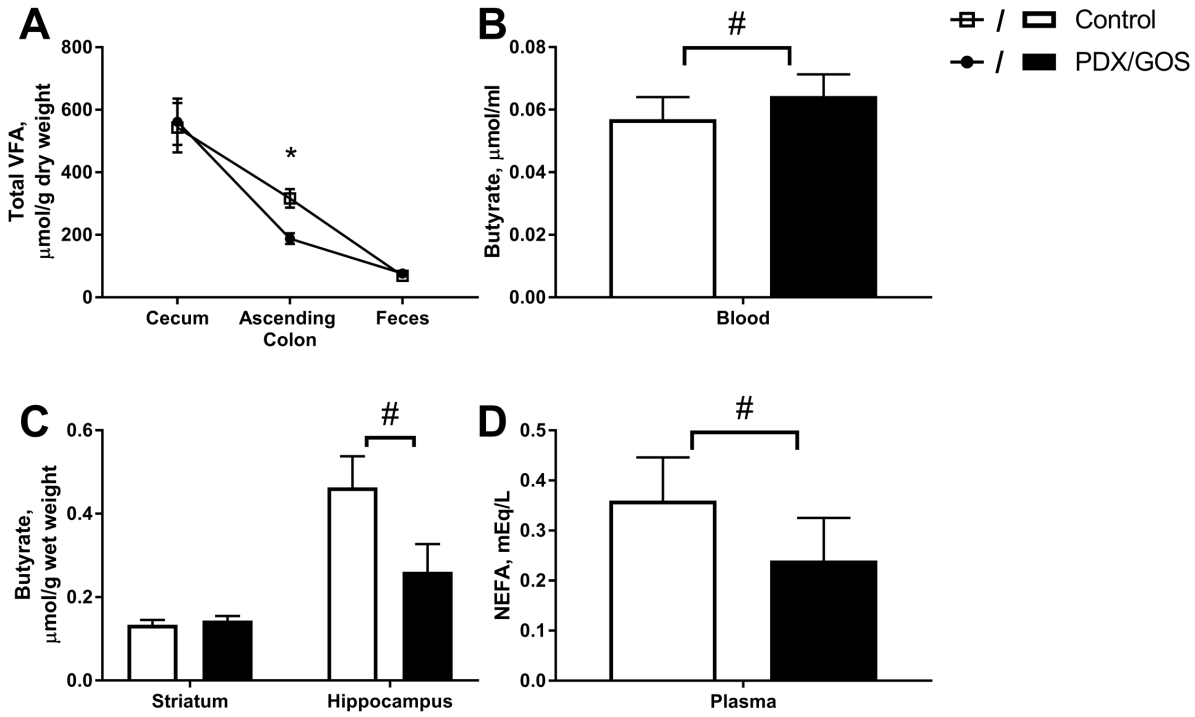

Figure 3.2: **A)** Pigs fed PDX/GOS displayed decreased concentrations of all VFA measured (acetate, propionate, butyrate, isovalerate, valerate, isobutyrate) in ascending colon samples, but not in cecal or fecal samples. **B–C)** While acetate was detected in both blood and brain, pigs fed PDX/GOS displayed higher butyrate in blood and lower butyrate in the hippocampus. **D)** Pigs fed PDX/GOS displayed lower plasma NEFAs than control pigs. * $p < 0.05$, # $p < 0.10$.

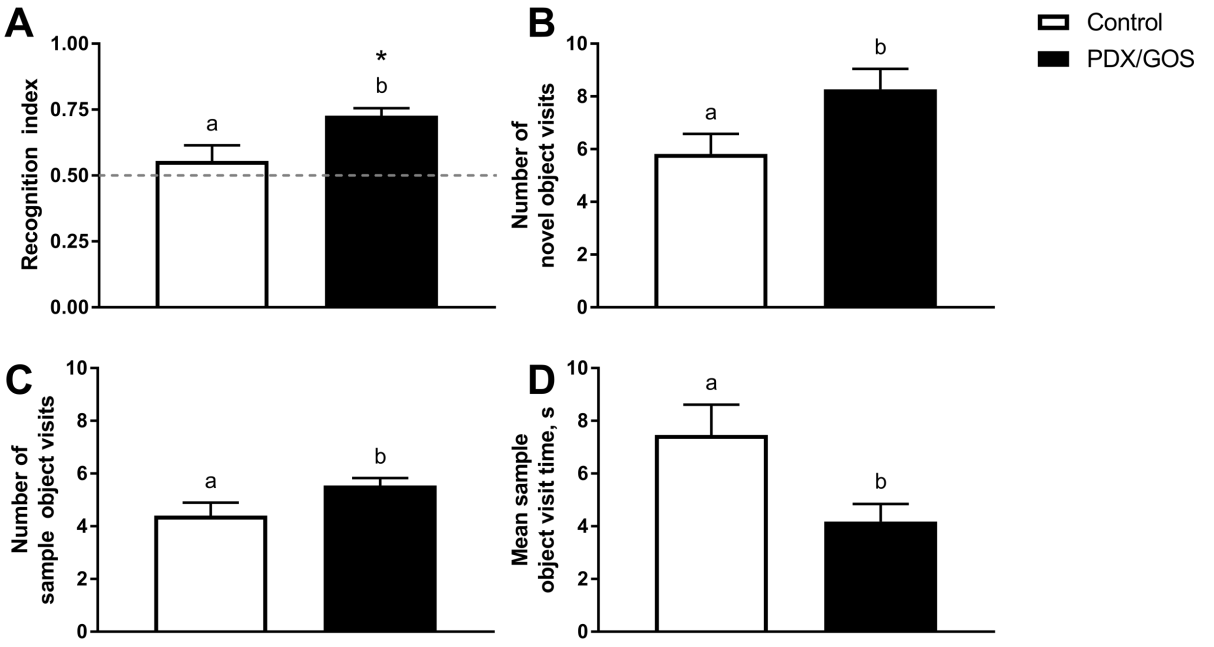

Figure 3.3: Effect of diet on exploratory behavior in the test trial of the NOR task. **A)** Pigs fed PDX/GOS were able to show a novelty preference (i.e. recognition index above 0.5 as indicated by the asterisk, $p < 0.001$) and had a greater recognition index than Control pigs. **B-D)** These pigs also visited the novel and sample object more frequently, but when visiting the sample object did so for a shorter duration per visit than pigs fed the control diet. Overall, these results indicate that pigs fed PDX/GOS exhibited greater exploration in response to novelty and demonstrated recognition memory for objects seen 45 hours prior. abMeans without a common superscript letter differ ($p < 0.05$).

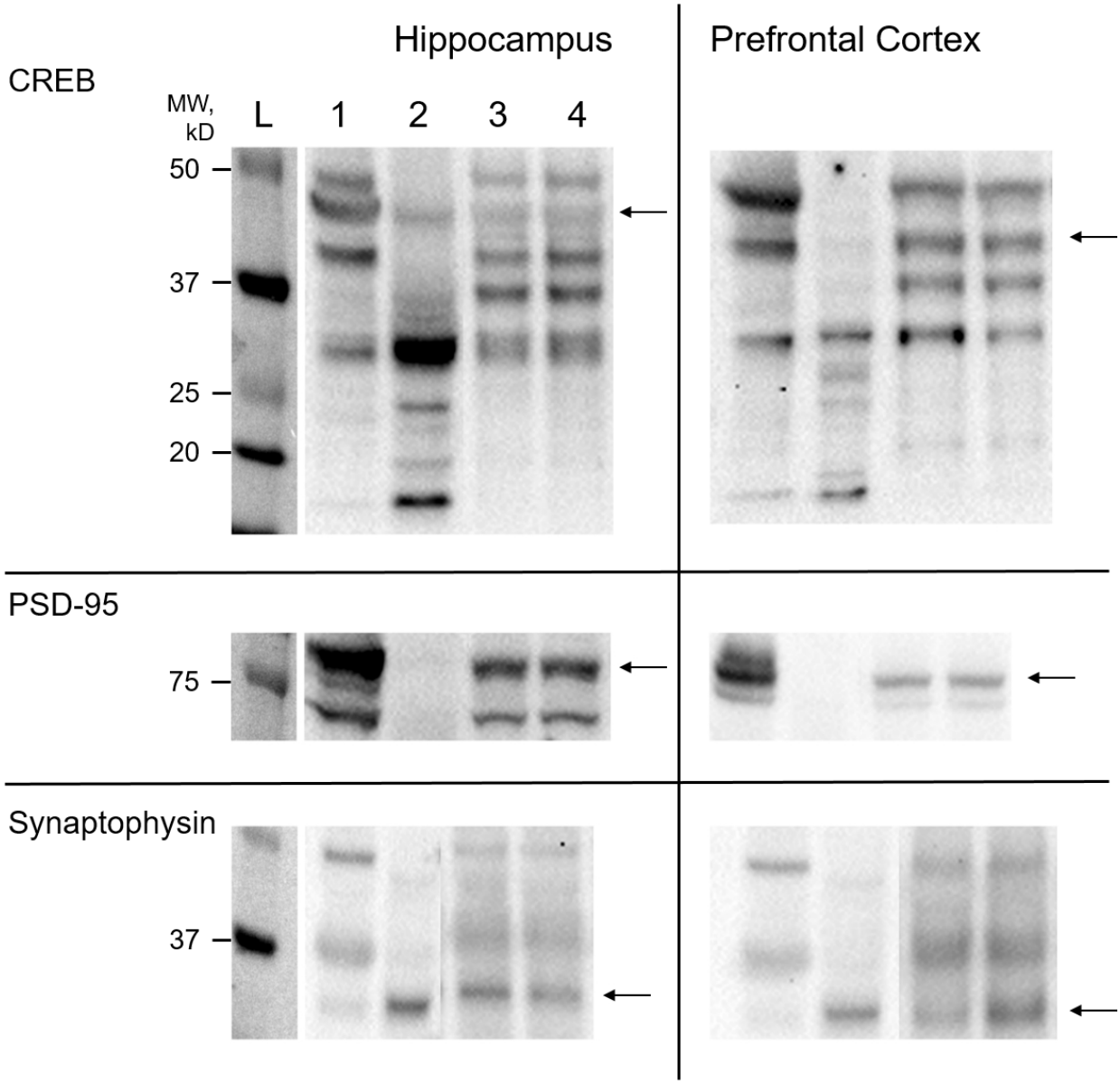

Figure 3.4: Representative image of western blots from hippocampal and medial prefrontal cortex tissues. Each panel depicts tissue from whole mouse brain (lane 1, positive control), pig liver (lane 2, negative control), PDX/GOS supplemented group (lane 3), control group (lane 4), and molecular weight standard (lane L). Multiple bands for all proteins were observed, the arrow indicates the band analyzed. These bands represent CREB, PSD-95, and synaptophysin, and were chosen by selecting the band at the molecular weight predicted by the antibody vendor

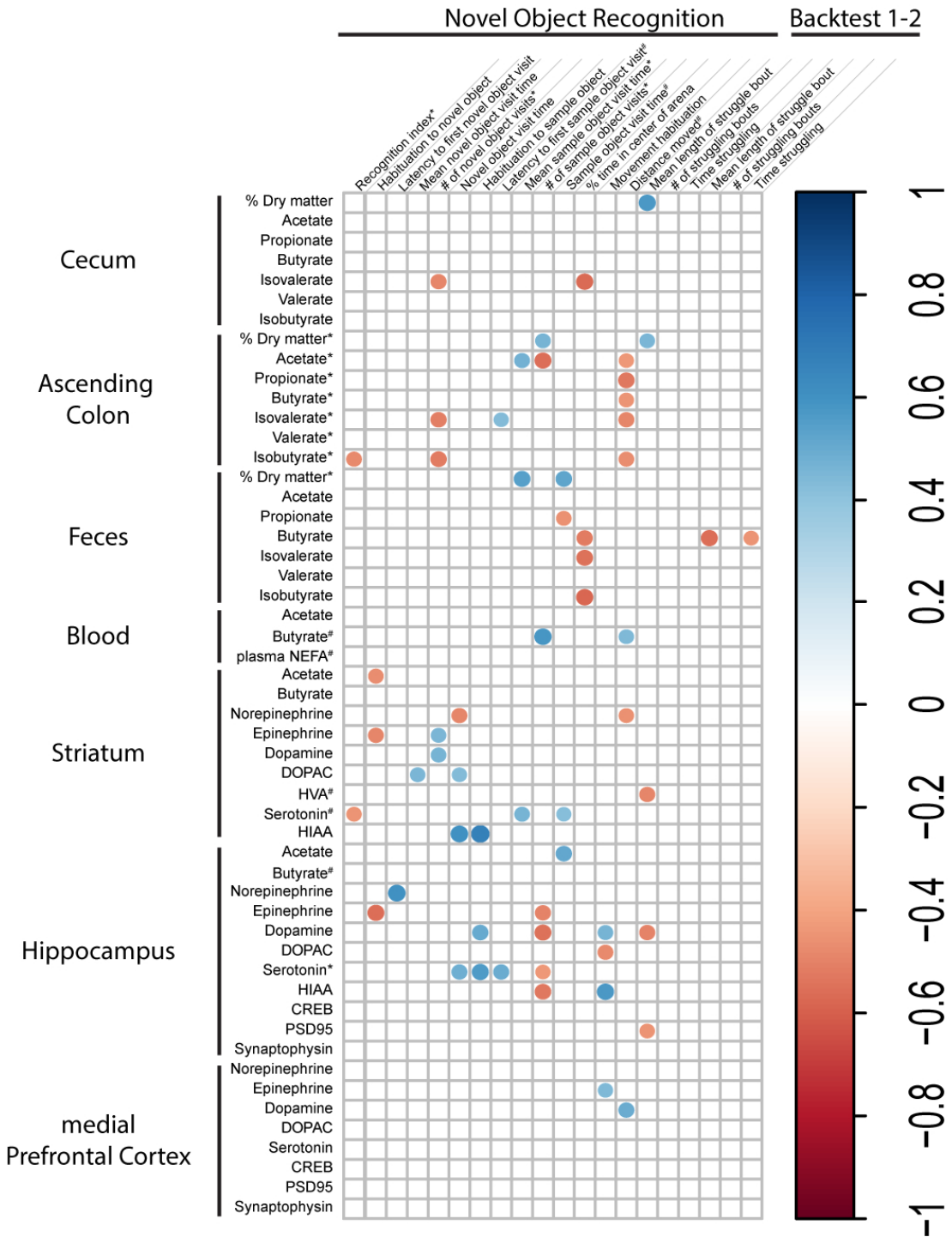

Figure 3.5: Correlation matrix for outcome variables, with intensity of colored dots referring to correlation strength. All NOR variables are from the test trial. Abbreviations: NEFA, non-esterified fatty acid; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; HIAA, 5-hydroxyindoleacetic acid; CREB, cAMP response element-binding protein; PSD95, post-synaptic density protein 95. Measures affected by diet as noted: * $p < 0.05$, # $p < 0.10$.

Chapter 4

Dietary sialyllactose does not influence recognition memory[1]

4.1 Abstract

Sialic acid (SA) is an integral component of gangliosides and signaling molecules in the brain and its dietary intake may support cognitive development. We previously reported that feeding sialyllactose, a milk oligosaccharide that contains SA, alters SA content and diffusivity in the pig brain. The present research sought to expand upon such results and describe the effects of feeding sialyllactose on recognition memory and sleep/wake activity using a translational pig model. Pigs were provided *ad libitum* access to a customized milk replacer containing 0 g/L or 380 g/L of sialyllactose from postnatal day (PND) 2–22. Beginning on PND 15, pigs were fitted with accelerometers to track home-cage activity and testing on the novel object recognition task began at PND 17. There were no significant effects of diet on average daily body weight gain, average daily milk intake, or the gain-to-feed ratio during the study (all $p \geq 0.11$). Pigs on both diets were able to display recognition memory on the novel object recognition task ($p < 0.01$) but performance and exploratory behavior did not differ between groups (all $p \geq 0.11$). Total activity and percent time spent sleeping were equivalent between groups during both day and night cycles (all $p \geq 0.56$). Dietary sialyllactose did not alter growth performance of young pigs, and there was no evidence that providing SA via sialyllactose benefits the development of recognition memory or gross sleep-related behaviors.

[1]Fleming, S.A., Chichlowski, M., Berg, B.M., Donovan, S.M., and Dilger, R.N. 2018. Dietary sialyllactose does not influence measures of recognition memory or diurnal activity in the young pig, *Nutrients*. 10(4). 395-407. The copyright owner has provided permission to reprint.

4.2 Introduction

Human milk has been shown to have numerous benefits in comparison to infant formula in stimulating the growth and development of gastrointestinal and immune systems (Ballard and Morrow, 2013). A recent meta-analysis suggests that breastfeeding promotes cognitive development (Horta et al., 2015), but the mechanisms and strength of the relationship are unclear (Girard et al., 2017). There is mounting evidence that components of human milk such as DHA (Hoffman et al., 2009), choline (Zeisel, 2004), and gangliosides (Palmano et al., 2015) support brain development, and emerging research suggests milk oligosaccharides (ranging from 3–32 monosaccharide units in length (Morrow et al., 2005)) may contribute to brain development as well (Bode, 2012). Some milk oligosaccharides may act as prebiotics (Bode, 2012), and we recently demonstrated that pigs fed a combination of prebiotics demonstrated increased exploratory behavior and improved recognition memory (Fleming et al., 2017). Milk oligosaccharides are a heterogeneous group of oligosaccharides with diverse functions largely related to immunity and gut physiology (Bode, 2012). The composition of human milk oligosaccharides is 10–20% sialylated (Bode, 2012). As sialic acid (SA) is present at relatively high concentrations in the brain as a part of gangliosides and signaling molecules that regulate neurodevelopment (Palmano et al., 2015), the impact of sialylated oligosaccharides such as sialyllactose (SL) is of interest as they may support brain development.

Supplementation with SA-containing ingredients, including complex milk lipids (Vickers et al., 2009), gangliosides (Liu et al., 2014), casein glycomacropeptide (cGMP) (Wang et al., 2007b), lactoferrin (Chen et al., 2015), and SL (Tarr et al., 2015), has been shown to improve cognition or alter stress-related behaviors. In a young adult mouse model investigating possible anxiolytic effects of SL, both 3' and 6' isomers reduced anxiety-related measures and restored performance to control levels when mice were introduced to a social stressor. Furthermore, SL attenuated stress-related sleep disruptions in adult rats (Chichlowski et al., 2017) and tended to increase performance on spatially based behavioral tasks (Sakai et al., 2006). A recent study demonstrated that pigs fed SL-supplemented formula for 21 days had greater total SA concentrations in the corpus callosum when fed milk containing 2 g/L of either 3'- or 6'-SL compared with pigs provided formula containing no SL or 4 g SL/L milk (Jacobi et al., 2016). Similarly, a previous study from our lab showed that of a range of doses of SL, ranging from 55–779 mg SL/L of formula, a moderate dosage of 429 mg SL/L increased free-to-bound hippocampal SA, reduced bound SA in the prefrontal cortex, and increased mean, axial, and radial diffusivity in the corpus callosum (Mudd et al., 2017b). Taken together with the findings of Jacobi and colleagues (Jacobi et al., 2016), these results show that feeding SL results in dose-dependent, structural, and region-specific increases in brain SA, but it remains to be shown whether this has functional consequences for behavior. Accordingly, our hypothesis in the present study was that supplementation with a moderate dosage of 380 mg SL/L would improve the performance of pigs on the novel object recognition task and influence measures of sleep/wake activity. We chose to supplement the diet at 380 mg SL/L, which is within the concentration range found in mature human milk (Ten Bruggencate et al., 2014).

4.3 Methods

4.3.1 Animals and Housing

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. Approval for this research project was verified on 3 March 2015 and is identified as IACUC 15034 at the University of Illinois Urbana-Champaign. Beginning on postnatal day (PND) 2, naturally farrowed, intact male pigs (n = 36) were artificially-reared through PND 22. The trial was completed in one replicate with 18 pigs per diet, selected from 9 litters to control for genetics (same sire and related dams between litters) and initial bodyweight. All pigs were provided a single subcutaneous 5 mL dose of *Clostridium perfringens* antitoxin C and D (Colorado Serum Company, Denver, CO, USA) on PND 2. If health status appeared compromised (i.e., diarrhea, lethargy, elevated body temperature), an additional 5 mL dose of *C. perfringens* antitoxin C and D was administered orally until symptoms resolved; a total of seven pigs were given additional doses of antitoxin. Housing, temperature, and lighting were conducted as described previously (Mudd et al., 2017b). Two pigs were euthanized prior to the conclusion of the study due to insufficient weight gain and failure-to-thrive (n = 1/diet). Data from the remaining 34 pigs (n = 17/diet) were used for subsequent analyses and are presented herein.

4.3.2 Dietary Groups

All diets were produced by Mead Johnson Nutrition (Evansville, IN, USA) using a proprietary blend of nutrients formulated to meet the nutritional needs of growing pigs. Pigs were provided one of two custom diets from PND 2–22. The control diet (Control) included docosahexaenoic acid (DHA, 87 mg/100 g milk replacer powder; DSM, Heerlen, The Netherlands), arachidonic acid (ARA, 174 mg/100 g milk replacer powder; DSM, Heerlen, The Netherlands), galactooligosaccharide (GOS, 1.0 g/100 g milk replacer powder; FrieslandCampina, Zwolle, The Netherlands), and polydextrose (PDX, 1.0 g/100 g milk replacer powder; Danisco, Terre Haute, IN, USA). The experimental diet (Sialyllactose) was formulated using the Control diet as the base and supplemented with bovine-derived modified whey enriched with SL (SAL-10; Arla Foods Ingredients, Aarhus, Denmark) to provide a final SL concentration of 190 mg SL/100 g milk replacer powder.

Milk replacer powder was reconstituted fresh each day at 200 g of dry powder per 800 g of water. At this reconstitution rate, all diets provided equal concentrations of DHA (174 mg/L), ARA (348 mg/L), and PDX/GOS (each at 2 g/L). The reconstituted milk replacers were formulated to contain 0 mg SL/L (Control) or 380 mg SL/L (Sialyllactose). Pigs were fed *ad libitum* using an automated milk replacer delivery system that dispensed milk from 10:00 to 06:00 the next day. Leftover milk from the previous day and individual pig bodyweights were recorded daily. The remaining volume of milk was subtracted from the initial volume provided to quantify milk disappearance following the 20-h feeding period, which will henceforth be referred

to as milk intake. Milk intake from PND 21 was omitted from analyses as pigs were fasted overnight prior to the end of study on PND 22. An electrolyte solution (Swine Bluelite, Tech Mix, Stewart, MN, USA) was provided to all pigs from PND 2–5 to help maintain electrolyte balance and avoid dehydration.

4.3.3 Behavioral Testing

4.3.3.1 Novel Object Recognition

The novel object recognition (NOR) task was used to assess object recognition memory. Testing consisted of a habituation phase, a sample phase, and a test phase. During the habituation phase, each pig was placed in an empty testing arena for 10 min each day for two days leading up to the sample phase. In the sample phase, the pig was placed in the arena containing two identical objects and given 5 min for exploration. After a delay of 48 h, the pig was returned to the arena for the test phase. During the test phase, the pig was placed in the arena containing one object from the sample phase as well as a novel object and allowed to explore for 5 min. Between trials, objects were removed, immersed in hot water with detergent and rubbed with a towel to mitigate odor, and the arena was sprayed with water to remove urine and feces. Objects chosen had a range of characteristics (i.e., color, texture, shape, and size); however, the novel and sample objects only differed in shape and size. Only objects previously shown to elicit a null preference were used for testing. Habituation trials began at PND 17 and testing on the NOR task began at PND 19. The amount of time exploring objects and distance moved was measured using a combination of automated procedures using Ethovision (Ethovision XT 11®, Noldus Information Technology, Wageningen, The Netherlands) and manual tracking (for a review of each measure assessed, see Fleming and Dilger (Fleming and Dilger, 2017)). The recognition index, the proportion of time spent with the novel object compared to total exploration of both objects, was used to measure recognition memory. A recognition index significantly above 0.50 demonstrates a novelty preference and, thus, recognition memory. Trials were removed from analyses if experimental/technical errors existed or pigs explored either object for less than 2 s during the sample or test trial. If pigs did not explore either object for greater than 2 s during the sample trial, they were also removed from analysis during the test trial regardless of performance. Two and five pigs provided the Control and SL diets, respectively, did not meet the above criteria and were removed from the final analysis (final n = 15, Control; n = 12, Sialyllactose).

4.3.3.2 Activity Analysis

Accelerometers (Actiwatch 2, Philips Respironics, Bend, OR, USA) were secured to collars and fastened around each pig's neck between PND 15 and 22 (n = 12 per diet) and were set to sample movement every 15 s. Only periods where full day and night cycles were recorded were used for analysis (PND 15 and 22 were omitted as collars were only on for part of the day, for a remaining total of six full cycles between PND 16 and 21). When pigs were found without collars, the collar was re-applied and the time was noted. After study

completion, home-cage video was used to verify that periods of complete inactivity were due to the loss of the collar, and these times were also removed from analysis. For the analysis of sleep/wake outcomes, specialized software (Actiware 6.0.7, Philips Respironics, Murrysville, PA, USA) was used to calculate a unique wake threshold value (used to determine if the pig was asleep or awake based on movement during 2-min periods before and after a single 15-s epoch) for each pig and quantify the total activity count and percent time asleep. Data were collected for six consecutive days and sleep outcomes were assessed as averages across that period.

A preliminary analysis was conducted to assess the validity of sleep scores in pigs from actigraphy data. Approximately one hour of activity data collected from six pigs was scored by actigraphy software as compared to the manual scoring of recorded video. Video was split into 15-s epochs, for a total of 247 epochs, and manually analyzed. If a pig was visually assessed as asleep for more than 50% of a single 15-s epoch, that epoch was classified as a "sleep" epoch. Epochs were chosen by selecting for periods of apparent transition between sleep and wakefulness as these are the most difficult to classify and appeared to be most variable between manual and automatic scoring methods.

4.3.4 Statistical Analysis

All data generated as part of this study were subjected to an analysis of variance (ANOVA) using the MIXED procedure of SAS Enterprise Guide 5.1 (SAS Institute Inc., Cary, NC, USA). Depending on the outcome, one of two statistical models was used: (1) data collected at a single time-point (e.g., average body weight gain over the entire study, performance in the NOR test trial) were analyzed by one-way ANOVA; and (2) data collected from the same animal on more than one occasion (e.g., diurnal activity) were analyzed using two-way repeated-measures ANOVA. Litter was included as a random effect in both statistical models. For NOR testing, a one-tailed t-test was conducted to assess if recognition index was greater than 0.5 (i.e., random chance). In all instances statistical significance was considered at $p < 0.05$.

4.4 Results

4.4.1 Diet Composition

Analytical assessment conducted after study completion showed levels of SL in the experimental diets were close to formulated levels (374 mg SL/L vs. 380 mg SL/L in the Sialyllactose diet). However, the Control diet contained 58 mg SL/L due to endogenous SL in the bovine milk ingredients. Energy, macronutrient, and micronutrient composition were comparable between Control and Sialyllactose diets (see Table 4.1 for analyzed nutrient composition).

4.4.2 Growth Performance and Health Status

No differences were observed for average daily body weight gain, average daily milk intake, or the feed efficiency ratio (i.e., gain-to-milk intake) between diets across the duration of the study (all $p \geq 0.11$, Table 4.2, Figure 4.1). Additionally, daily health checks revealed low incidence of loose stool in pigs and no differences in pig health status or compliance to consume experimental dietary treatments. Thus, all pigs remained healthy throughout the study duration and both dietary treatments were equally well tolerated by pigs as evident in the observed trajectory of body weight gain.

4.4.3 Novel Object Recognition

Regardless of dietary treatment, all pigs were able to display recognition memory in the NOR test trial ($p < 0.01$, Table 4.3). However, there were no differences between dietary treatment groups for measures of exploratory behavior, most notably time spent investigating objects, number of object visits, and mean time spent per object visit (all $p \geq 0.11$, Table 4.4). Although some pigs were removed due to non-compliance, ultimately our study was powered to capture an effect size of 0.89 with a power of 0.80 when evaluating differences in the NOR recognition index.

4.4.4 Activity Analysis

Validation of the automated scoring method (i.e., computer-assisted analysis of actigraphy data) against the manual scoring of home-cage video was performed. A chi-square test for equality of two proportions showed that automated and manual scoring methods were not different ($p = 0.065$), with the automated scoring being only 7% more likely to score an epoch as "sleep" and less likely to score an epoch as "awake". Therefore, with the validation of the automated actigraphy data scoring method complete, all sleep/wake activity reported herein was generated using the automated software-based method. In general, there was no significant main effect of diet or interaction effect of diet by cycle for total activity or percent time asleep (all $p \geq$) 0.56, Table 4.5). While intuitive, total activity counts and time asleep were both influenced by cycle (i.e., day vs. night; both $p < 0.01$).

4.5 Discussion

Sialyllactose is one of several sources of SA, and comparisons of mature human and porcine milk demonstrate that the SA content of human milk is much greater than that of porcine milk. Mature human milk provides approximately 500 mg SA/L milk (Martín-Sosa et al., 2004), porcine milk contains approximately 10 mg SA/L milk (Mudd et al., 2016c), and infant formula falls between 65–290 mg SA/L milk (Spichtig et al., 2010) (for a thorough review of SA content of milk and other food products, see Röhrig et al. (Röhrig et al., 2017)). In this study, a dose of 380 mg SL/L was tested as a previous study reported that this dose was

most effective at eliciting changes in SA content and diffusivity in the brain (Mudd et al., 2017b). This dose is well below the SL dose that was shown by Jacobi et al. (Jacobi et al., 2016) to enrich corpus callosum and hippocampal SA content (Jacobi et al., 2016), but is within the range found in mature human milk (Ten Bruggencate et al., 2014). Here, the impact of dietary SL on growth, recognition memory, exploratory behavior, and diurnal activity was investigated, but no impact of diet was observed for any measure.

Pigs fed an SL-supplemented diet did not display altered sleep behavior, whereas a past report demonstrated that SL attenuated disruptions to sleep architecture after exposure to an acute inescapable stress (Chichlowski et al., 2017). An important distinction from past research is that diurnal activity was measured in a minimally stressful environment, whereas SL may provide a neuroprotective effect that is only observed in the context of a more extreme stressor. Additionally, Chichlowski et al. (Chichlowski et al., 2017) used electroencephalography (EEG), allowing the experimenters to more accurately quantify the timing, stage, and quality of sleep. Although both treatment groups in our study had similar activity during the day and night, differences in the quality of sleep may have been observed if assessments were made using EEG.

While different doses and/or longer duration of SL administration may have produced a cognitive benefit, it is possible that supplemental SA (via SL) was not required in the behavioral paradigm under which the pigs were assessed. Active learning increases the expression of mRNA for the enzyme (GNE) critical for regulating SA biosynthesis by 2- to 3-fold in the hippocampus and liver of pigs (Wang et al., 2007a). As the NOR task makes use of spontaneous behavior rather than operant conditioning the cognitive load required to learn a rule was not present, and there may not have been a physiological demand for increased SA utilization in the brain. Our data show that dietary SL supplementation did not provide a cognitive benefit as assessed by NOR, which conflicts with previous work that showed young pigs exhibit cognitive benefits from dietary SA supplementation when using behavioral tasks dependent on operant conditioning (Wang et al., 2007b, Chen et al. (2015)). Moreover, these results may reflect the presence of prebiotics in both the control and experimental diet. We previously reported that pigs fed milk replacers containing the prebiotics PDX and GOS demonstrated increased exploratory behavior and improved recognition memory using the same behavioral paradigm (i.e., the NOR task with a delay of 48 h) (Fleming et al., 2017). Together with evidence that piglets are capable of performing the NOR task at younger ages using shorter delays (Fleming and Dilger, 2017), we believe the difficulty of the task was appropriate. Rather, the potential cognitive benefits from SL may have been masked by the inclusion of PDX and GOS in each diet.

To our knowledge, few studies have evaluated the ability of dietary SL to affect behavior and cognition. Tarr and colleagues (Tarr et al., 2015) found that dietary SL attenuated stressor induced anxiety-like behaviors in rats and preliminary data suggests that SL may prevent stress-induced alterations in sleep architecture (Chichlowski et al., 2017). However, another report in rats demonstrated that feeding SL only produced a non-significant trend towards improved cognition on spatial tasks (Sakai et al., 2006). The majority of data suggesting dietary SL may be beneficial for cognitive development come indirectly from

studies that investigated other SA-containing ingredients such as gangliosides (Vickers et al., 2009, Liu et al. (2014), Xu and Zhu (2005)), cGMP (Wang et al., 2007b), and lactoferrin (Chen et al., 2015). Each of these ingredients vary in structure and function but are common in that they contain SA. Gangliosides are sialylated glycosphingolipids highly concentrated in the brain (Palmano et al., 2015), cGMP is an SA-enriched peptide released during the formation of cheese from the protein kappa-casein (Neelima et al., 2013), and lactoferrin is an iron binding glycoprotein enriched in SA with various functions related to iron metabolism (Levay and Viljoen, 1995).

Gangliosides contribute 75% of conjugated SA in the brain where they play a critical role in functions such as synaptic transmission, plasticity, neurogenesis, synaptogenesis, cell proliferation, and cell differentiation (Palmano et al., 2015). Exogenous, but not dietary, gangliosides and SA appear to be effective at promoting cognition in adult or aging models (Fagioli et al., 1992, Silva et al. (1997), Fong et al. (1997), Silva et al. (2000), Popov et al. (1989)) and deficit (i.e., drug-induced amnesia, cortical lesions, malnourishment) models (Silva et al., 1999, Glasier et al. (1999), Morgan and Winick (1980)). The impact on young, normal animals is mixed, but ganglioside and SA administration have shown both positive and neutral effects on cognition (Fong et al., 1997, Wainwright et al. (2007)). These studies provided preliminary evidence that exogenous SA improves cognition; however, there is less evidence that dietary gangliosides improve cognition in normative models (i.e., gangliosides are provided at physiological concentrations via the diet to healthy animals during typical development).

Male and female pigs fed formula containing a mix of 0.8% or 2.5% phospholipids and gangliosides displayed fewer errors in a spatial T-maze test compared with controls and had larger brain weights. Furthermore, volumes of several brain regions including the internal capsule, putamen, and thalamus appeared sensitive to supplementation (Liu et al., 2014). As has been discussed for SA, the cognitive effects of dietary ganglioside supplementation appear dependent on dosage and behavioral task employed. Rats provided complex milk lipid in doses of 1.0% but not 0.2% exhibited greater behavioral performance in the novel object recognition and Morris water maze, but no improvement in operant conditioning tasks (Vickers et al., 2009). A subsequent report from the same group demonstrated that even lower doses of 0.05% and 0.01% had no effect on operant learning, and spatial or recognition memory (Gustavsson et al., 2010). In a separate study, dietary gangliosides fed to children with cerebral palsy for 3 months elicited improved muscle tension, limb function, language ability, and intelligence (Xu and Zhu, 2005). Due to the use of a developmentally appropriate preclinical model for the human infant, this study provides strong evidence that gangliosides contribute to not only cognition but also motor function and development. Taken together these data suggest that, provided the correct dosage, gangliosides fed alone or as part of a complex milk lipid may have the capacity to promote cognitive development.

Young pigs supplemented with SA via cGMP from PND 3–35 displayed dose-dependent increases in performance in the radial arm maze, with those provided the most cGMP completing the difficult

version of the task with the fewest mistakes. All groups fed cGMP had enriched protein-bound, but not ganglioside-bound, SA in the frontal cortex. Additionally, pigs fed the highest amount of cGMP had increased levels of *ST8SIAIV*, a polysialyltransferase important in SA metabolism. After a correlation analysis, it was revealed that sialyltransferase activity in the frontal cortex correlated inversely with number of mistakes on the behavioral task, with pigs exhibiting lower sialyltransferase activity making more mistakes. Despite this correlation, there were no dietary effects on sialyltransferase activity, suggesting sialyltransferase activity may not be involved in the mechanism by which performance was improved. In a later study by the same group, pigs supplemented with lactoferrin from PND 3–38 were found to have increased performance on the radial maze. More pigs in the treatment group were able to complete both easy and difficult versions of the task, with the pigs provided lactoferrin making fewer mistakes on the difficult version (Chen et al., 2015). Gene microarray data in hippocampal tissue revealed that pigs fed lactoferrin had upregulated expression of genes in the brain-derived neurotrophic factor (BDNF) neurotrophic signaling pathway, affecting genes related to organization of the cytoplasm, cytoskeleton, growth of neurites, and anxiety (Chen et al., 2015). The finding that the provision of dietary lactoferrin influenced the expression of genes related to anxiety suggested that this protein may decrease anxiety, which aligns with the previously discussed results from Tarr et al. (Tarr et al., 2015), wherein mice provided SL demonstrated attenuated anxiety when introduced to a social stressor.

Overall, few studies have evaluated the impact of SL on cognition and behavior. However, there is evidence that SA-containing ingredients positively influence cognitive performance, but making cross-sectional comparisons is confounded by the use of several different SA-enriched ingredients. While containing SA, SL, gangliosides, cGMP, and lactoferrin differ vastly in structure and function, contributing to the variation between study results.

Although our intervention coincided with a significant portion of brain growth in the pig, our investigation may have been limited by the duration of the trial, which may not have allowed sufficient time for SL to confer cognitive benefits. As discussed, the novel object recognition task may not reflect a context wherein supplemental SA is beneficial, and comparisons between spontaneous and operant behavior may elucidate the conditions that lead to increased neural requirements for SA. While clinically translatable, the use of actigraphy instead of EEG did not allow the examination of neural activity during sleep and the quantification of sleep stages, thus our measures were only a gross representation of sleep activity. Lastly, although the goal of this study was to evaluate the efficacy of 380 mg SL/L at supporting cognitive development, we cannot exclude the possibility that a higher supplementation dose or longer supplementation period may have elicited cognitive benefits.

4.6 Conclusions

While there are several reports that SA-containing ingredients may influence cognitive development, we found no evidence that bovine-derived dietary SL provided at 380 mg SL/L was effective at altering recognition memory or sleep-related activity.

4.7 Supplemental Data

Supplemental data for this study can be accessed through the journal *Nutrients* at https://doi.org/10.3390/nu10040395.

4.8 Tables

Table 4.1: Analyzed nutrient composition of the diet.

Nutrient per Liter	Control	Sialyllactose
Sialyllactose, mg	58	374
Energy and Macronutrients		
Total calories, kcal	1049	1020
Carbohydrate, g	57	58
Fat, g	64	60
Protein, g	61	62
Minerals		
Calcium, mg	2233	2178
Chlorine, mg	1141	1158
Copper, μg	1640	1505
Iodine, μg	274	271
Iron, mg	19	19
Magnesium, mg	227	241
Manganese, μg	2305	2159
Phosphorus, mg	1621	1673
Potassium, mg	2255	2349
Selenium, μg	65	68
Sodium, mg	1708	1708
Zinc, mg	17	17
Vitamins and other nutrients		
Vitamin A, IU	4572	4112
Vitamin D3, IU	761	795
Vitamin E, IU	30	31
Vitamin K, μg	321	362
Thiamin, μg	1322	1588
Riboflavin, μg	2608	2780
Niacin, μg	13366	11132
Vitamin B6, μg	1210	1414
Folic Acid, μg	211	237
Vitamin B12, μg	6	7
Pantothenic Acid, μg	9216	8170

Table 4.1: Analyzed nutrient composition of the diet. *(continued)*

Nutrient per Liter	Control	Sialyllactose
Biotin, μg	74	74
Choline, mg	352	394
Polydextrose, g	1.8	1.9
Galactooligosaccharide, g	2.1	1.7
Arachidonic Acid, mg	318	288
Docosahexaenoic Acid, mg	155	141

Table 4.2: Growth performance.[*†]

Measure[‡§]	Control	Sialyllactose	Pooled SEM	p-value
ADG, g/day	311	306	14	0.69
ADMI, g milk/day	1,220	1,347	62	0.11
ADMI, g solids/day	244	269	12	0.11
G:F, g BW:kg milk	255	234	11	0.16

[*] Abbreviations: SEM, standard error of the mean; BW, body weight; kg, kilogram; ADG, average daily body weight gain; ADMI, average daily milk intake; G:F, gain-to-feed ratio (i.e., feed efficiency).
[†] Data analyzed by mixed model ANOVA.
[‡] Calculations reflect a milk reconstitution rate of 20% solids.
[§] $n = 17$ per diet

Table 4.3: Recognition memory performance.[*†]

Diet	n	Mean	Pooled SEM	p-value
Control	15	0.65	0.046	< 0.01
Sialyllactose	12	0.66	0.047	< 0.01

[*] Abbreviations: n, sample size; SEM, standard error of the mean.

[†] Pigs were tested on the novel object recognition task using a delay of 48 hours. A one-sample t-test was conducted to assess recognition index greater than 0.50 as an indication of recognition memory

Table 4.4: Exploratory behavior in the NOR task.[*†]

	Control		Sialyllactose			
Measure	n	Mean	n	Mean	Pooled SEM	p-value
Recognition index	15	0.66	12	0.65	0.05	0.94
Novel object visit time, s	15	56.63	12	42.05	7.77	0.18
Number of novel object visits	15	8.33	12	6.78	1.07	0.25
Mean novel object visit time, s	15	6.19	11	6.41	1.12	0.88
Latency to first novel object visit, s	15	25.46	12	25.32	9.31	0.99
Habituation towards the novel object, s/min	15	-1.60	12	-0.69	1.25	0.59
Sample object visit time, s	14	28.27	12	22.45	6.45	0.50
Number of sample object visits	15	4.77	12	4.62	0.56	0.84
Mean sample object visit time, s	15	6.18	12	5.51	1.34	0.71
Latency to first sample object visit, s	14	24.08	12	14.25	7.15	0.31
Habituation towards the sample object, s/min	14	-2.68	12	-1.78	0.73	0.35
Total object visit time, s	15	82.52	12	70.35	13.51	0.47
Mean object visit time, s	15	7.10	12	6.11	1.22	0.55
Number of object visits	15	13.13	12	11.42	1.34	0.31
Latency to first object visit, s	15	9.48	12	13.50	4.97	0.55
Habituation towards both objects, s/min	15	-4.32	12	-1.76	1.39	0.19
Total distance moved, m	15	2.43	11	2.11	0.19	0.11
Time spent in the center of the arena, %	15	58.76	12	56.73	6.95	0.80

[*] Abbreviations: n, sample size; SEM, standard error of the mean; NOR, novel object recognition.

[†] Pigs were tested on the novel object recognition task using a delay of 48 hours. A one-sample t-test was conducted to assess recognition index greater than 0.50 as an indication of recognition memory. Data were analyzed via mixed model ANOVA

Table 4.5: Diurnal activity.[*†]

	Control				Sialyllactose							
	Day		Night		Day		Night			p-value		
Measure	n	Mean	n	Mean	n	Mean	n	Mean	Pooled SEM	Diet	Cycle	Int
Total activity count	65	1.6×10^5	70	6.9×10^4	67	1.7×10^5	72	6.9×10^4	7.0×10^3	0.64	< 0.01	0.56
Time asleep, %	65	67.47	70	84.9	71	67.43	72	85.63	0.97	0.61	< 0.01	0.58

[*] Abbreviations: n, sample size; SEM, standard error of the mean; Int, interaction

[†] Data were collected form 12 pigs per diet over a six-day period and analyzed via mixed model ANOVA

4.9 Figure

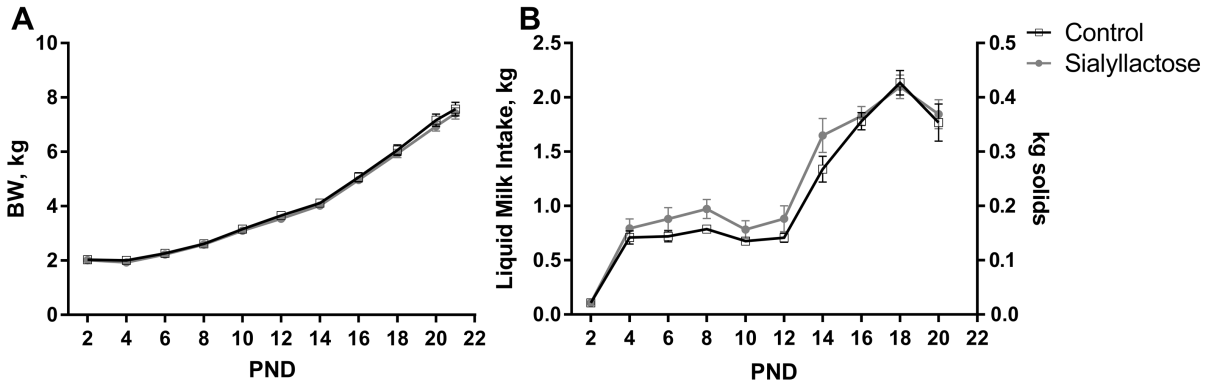

Figure 4.1: **A)** Body weight (BW) and **B)** liquid milk intake during the trial. No differences in average daily body weight gain, average daily milk intake, or the feed efficiency ratio (i.e., body weight gain:feed intake ratio) were observed between groups ($p \geq 0.11$). Data for milk intake on postnatal day (PND) 22 are not shown as piglets were fasted overnight prior to the end of study.

Chapter 5

Dietary oligofructose alone or in combination with 2'fucosyllactose differentially improves recognition memory and alters brain structure and hippocampal mRNA expression[1]

5.1 Abstract

Mounting evidence suggests dietary oligosaccharides promote brain development. This study assessed the capacity of oligofructose (OF) alone or in combination with 2'fucosyllactose (2'FL) to alter recognition memory, structural brain development, and hippocampal gene expression. Beginning on postnatal day (PND) 2, male pigs received one of three milk replacers formulated to contain OF, OF + 2'FL, or no oligosaccharides (CON). Pigs were tested on the novel object recognition task using delays of 1- or 48-h at PND 22. At PND 32-33, magnetic resonance imaging (MRI) procedures was used to assess structural brain development and hippocampal tissue was collected for analysis of mRNA expression. Pigs that consumed the OF diet demonstrated increased recognition memory after a 1-h delay whereas those consuming diets containing OF + 2'FL displayed increased recognition memory after a 48-h delay. Pigs fed OF or OF + 2'FL exhibited a larger relative volume of the olfactory bulbs compared with CON pigs. Provision of OF or OF +

[1]Dietary oligofructose alone or in combination with 2'fucosyllactose differentially improves recognition memory and alters brain structure and hippocampal mRNA expression, *Scientific Reports*. Under review.

2'FL altered gene expression related to dopaminergic, GABAergic, cholinergic, cell adhesion, and chromatin remodeling processes. Collectively, these data indicate that dietary OF and OF + 2'FL differentially improve cognitive performance and affect olfactory bulb structural development and hippocampal gene expression.

5.2 Introduction

While their ability to stimulate gut bacteria and prebiotic activity has been known for some time, it is becoming increasingly evident that oligosaccharides (OS) act through unknown mechanisms to stimulate brain development. Oligofructose (OF), also known as fructooligosaccharide (FOS), is an OS of vegetable origin, commonly found in foods such as asparagus, artichokes, onions, and wheat (Clevenger et al., 1988). OF is non-digestible and readily fermentable by *Bifidobacterium* spp. and *Bacteroides* spp., but not by potentially pathogenic bacteria such as *Escherichia coli* and *Clostridium perfringens* (Rivero-Urgell and Santamaria-Orleans, 2001). Infants consuming formula containing OF display increased fecal *bifidobacteria* and *bacteroides*, decreased fecal *Escherichia coli* and *enterococci*, and increased stool frequency (Costalos et al., 2008, Kapiki et al. (2007)). Consumption of OF has also been shown to alter the expression of brain derived neurotrophic factor (BDNF) and N-methyl-D-aspartate (NMDA) receptor subunits in the rodent brain5 and improve cognitive function in a rodent model of Alzheimer's disease (Yen et al., 2017, Chen et al. (2017)).

Human milk contains a heterogenous group of OS that have demonstrated benefits for immune and intestinal function and are hypothesized to promote brain development (Ballard and Morrow, 2013, Bode (2012)). The concentration and diversity of these human milk oligosaccharides (HMO) is un-matched by other mammals (Urashima et al., 2001), and these OS are specific to milk as opposed to non-milk OS such as OF. This has significant implications for infants consuming bovine-milk-based infant formulas, as bovine milk contains up to a hundred times fewer and less diverse OS (Bode, 2012). Infants consuming formula containing 2'fucosyllactose (2'FL) and lacto-N-neotetraose report fewer incidences of respiratory illness (Puccio et al., 2017). Evidence from rodent studies suggests HMO such as sialyllactose and 2'FL may improve response to stress (Tarr et al., 2015) and learning and memory (Vázquez et al., 2015, Vazquez et al. (2016)), respectively. Both sialyllactose and 2'FL contain monosaccharides (i.e., sialic acid and fucose, respectively) that are known glycoconjugates in the brain (Palmano et al., 2015, Sukumar et al. (1980)). Whether sialyllactose and 2'FL impact the brain in large part due to their sialic acid or fucose content is unclear. Sialic acid, alone or as part of a ganglioside, is known to promote cognition (Fagioli et al., 1990, Silva et al. (1996), Popov et al. (1989)). Similarly, fucose has been shown to accrete in glycoproteins after a passive avoidance task in chicks (Sukumar et al., 1980), and impairing fucosylation in the rat hippocampus impairs retention during discrimination tasks (Jork et al., 1986). Yet, Vazquez et al. demonstrated that intact 2'FL, and not fucose, promotes hippocampal long-term potentiation (Vazquez et al., 2016).

Alluding to the probability that intact OS and not their active monosaccharide components are required

for promoting cognition is the evidence that OS such as OF, galactooligosaccharide (GOS), and chitosan oligosaccharide have been shown to benefit cognition in various animal models and species (Chen et al., 2017, Fleming et al. (2017), Schmidt et al. (2015), Jia et al. (2016)). This is significant as some formulas contain OF or GOS (Bertelsen et al., 2016), yet these OS are not found in human milk. Whether HMO provide a cognitive benefit greater than that of non-human milk OS is an important question as infants relying on infant formulas as their sole source of nutrition are typically not provided the level and diversity of HMO that are present in human milk.

We chose to use the neonatal piglet as animal model due to their similarities to the human regarding gastrointestinal physiology (Odle et al., 2014) and brain development (Mudd and Dilger, 2017). As the majority of studies assessing the efficacy of OS to promote brain development have been conducted in rodent models, this study evaluated whether such effects can be replicated in an animal model closer to humans. As previously mentioned, it is unclear if HMO provide a cognitive benefit in addition to formula already containing non-HMO OS. Thus, the objective of this study was to assess the impact of dietary OF alone or combined with 2'FL on recognition memory, hippocampal gene expression, and structural development of the brain using the pig as an animal model.

5.3 Methods

5.3.1 Animals and Housing

All animal care and experimental procedures were in accordance with the National Research Council Guide for Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. Thirty-six intact male pigs (1050 Cambro genetics) were naturally farrowed and allowed colostrum consumption for up to 48 h before transport to the Piglet Nutrition & Cognition Laboratory at the University of Illinois at Urbana-Champaign. Pigs were artificially reared from postnatal day (PND) 2 until PND 33. This study was conducted using six independent cohorts (n = 2 pigs per dietary treatment in each cohort), with pigs selected to control for genetics and initial body weight within cohort. All pigs were housed in master caging units that contained six individual stainless-steel cages (L × W × H of 87.6 × 88.9 × 50.8 cm) with clear, polycarbonate facades on three sides of the cage and vinyl-coated, expanded-metal flooring (Tenderfoot®, Minneapolis, MN). The master unit was designed such that there were three separate levels each with two individual pig cages on each level. Thus, pigs on each level shared a common wall containing holes to permit pigs to see, smell, hear, and minimally touch one-another. A towel and toy were included in each cage to provide enrichment, all pigs were removed from cages and allowed to socialize for approximately 30 min each day, and all pigs were allowed *ad libitum* access to water at all times.

All pigs were reared in the same room with ambient temperature maintained between 27°C and 29°C and a 12-h light/dark cycle maintained from 0600 to 1800 h. Prior to placement in the artificial rearing system, pigs

were administered 5.0 mL of *Clostridium perfingens* antitoxin C + D per the manufacturer's recommendations (Colorado Serum Company, Denver, CO, USA). At study conclusion (PND 33), pigs were anesthetized using a telazol:ketamine:xylazine solution (50.0 mg tiletamine plus 50.0 mg of zolazepam reconstituted with 2.50 mL ketamine [100 g/L] and 2.50 mL xylazine [100 g/L]; Fort Dodge Animal Health) by intramuscular injection at 0.03 mL/kg bodyweight. After anesthetic induction, pigs were euthanized via intracardiac administration of sodium pentobarbital (86.0 mg/kg of body weight; Euthasol, Virbac Animal Health, Fort Worth, TX).

5.3.2 Dietary Treatments

All researchers involved with conducting the study and acquiring and analyzing study results remained blind to dietary treatment identity until final data analyses were complete. Pigs (n = 12 per diet) were provided milk replacers reconstituted at 200 g of dry powder per 800 g of water. Reconstituted diets were formulated to contain 0 g/L OF + 0 g/L 2'FL (control [CON], ProNurse® Specialty Milk Replacer, Purina Animal Nutrition, Gray Summit, MO), 5.22 g/L OF + 0 g/L 2'FL (OF), or 5.22 g/L OF + 0.98 g/L 2'FL (OF + 2'FL). All diets were formulated to contain supplemental lactose to balance the amount carbohydrate between diets: CON, OF, and OF + 2'FL contained 13.86, 8.64, and 7.66 g/L lactose, respectively.

Post-hoc analysis of the diets revealed actual OF concentrations at 0, 3.62, and 3.41 g/L and 2'FL at 0, 0, and 1.12 g/L in the CON, OF, and OF + 2'FL diets, respectively. These values correspond to an inclusion rate of OF at 0, 1.81, and 1.71%, and 2'FL at 0, 0, and 0.56% oligosaccharide in the diet on a weight OS for weight powder basis. Given the diets contained lactose in the base formula, supplemental lactose could not be quantified after formulation. Pigs received small volumes (approximately 500 mL) of experimental diets on the day of arrival to the rearing facility to allow for adjustment to the milk replacer prior to the standard feeding regimen. Pigs were fed at a rate of 285 mL and 325 mL of reconstituted diet per kg bodyweight from PND 2-6 and PND 7-33, respectively. Individual pig bodyweight was recorded daily to determine the volume of milk to be dispensed to individual animals throughout the day. Meals were administered 10 times a day, approximately every 100 min, between 1100 and 0400 h using an automated feeding system.

5.3.3 Behavior

Pigs were tested on the novel object recognition (NOR) task using two different delays to assess intermediate and long-term recognition memory. Testing consisted of a habituation phase, a sample phase, and a test phase. During the habituation phase, each pig was placed in an empty testing arena for 10 min each day for two days leading up to the sample phase. In the sample phase, the pig was placed in the arena containing two identical objects and given 5 min for exploration. After a delay of 1- or 48-h the pig was returned to the arena for the test phase of the NOR task. During the test phase, the pig was placed in the arena containing one object from the sample phase and a novel object and allowed to explore for 5 min. Between trials, objects were removed, immersed in hot water with detergent, and rubbed with a towel to mitigate odor and

the arena was sprayed with water to remove urine and feces. Objects chosen had a range of characteristics (i.e., color, texture, shape, and size), however the novel and sample objects only differed in shape and size. Only objects previously shown to elicit a null preference were used for testing (Fleming and Dilger, 2017). Task order was counterbalanced between replicates. Habituation trials began at PND 22 and testing on the sample phase began on PND 24. Recognition index, or the proportion of time spent with the novel object compared to total exploration of both objects, was used to measure recognition memory. A recognition index significantly above 0.50 demonstrates a novelty preference and thus recognition memory.

5.3.4 Magnetic Resonance Imaging

All pigs underwent MRI procedures at PND 32 or 33 at the Beckman Institute for Advanced Science and Technology Biomedical Imaging Center using Siemens MAGNETOM Trio 3T equipment with a Siemens 32-channel head coil. Each pig underwent imaging protocols only once, and scans for each cohort of pigs were completed all on the same day. The pig neuroimaging protocol included three magnetization prepared rapid gradient-echo (MPRAGE) sequences and diffusion tensor imaging (DTI) to assess brain macrostructure and microstructure, respectively, as well as magnetic resonance spectroscopy (MRS) to obtain brain metabolite concentrations. In preparation for MRI procedures, anesthesia was induced using an intramuscular injection of telazol (50.0 mg of tiletamine plus 50.0 mg of zolazepam reconstituted with 5.0 DI water; Zoetis, Florham Park, NJ) administered at 0.07 mL/kg bodyweight, and maintained with inhalation of isoflurane (98% O_2, 2% isoflurane). Pigs were immobilized during all MRI procedures. Visual observation of each pig's well-being, as well as observations of heart rate, PO_2 and percent of isoflurane were recorded every 5 min during the procedure and every 10 min post-procedure until animals recovered. Total scan time for each pig was approximately 60 min. Imaging techniques are briefly described below.

5.3.4.1 Structural MRI

A T_1-weighted magnetization-prepared rapid gradient echo (MPRAGE) sequence was used to obtain anatomic images of the pig brain with a 0.7 mm isotropic voxel size. Three repetitions were acquired and averaged using SPM8 in Matlab 8.3, and brains were manually extracted using FMRIB Software Library (FSL) (FMRIB Centre, Oxford, UK). The following sequence specific parameters were used to acquire T_1-weighted MPRAGE data: repetition time (TR) = 1900 ms; echo time (TE) = 2.49 ms; 224 slices; field of view (FOV) = 180 mm; flip angle = 9°. Methods for MPRAGE averaging and manual brain extraction were previously described (Mudd et al., 2016b). All data generated used a publicly-available population-averaged pig brain atlas (http://pigmri.illinois.edu)(Conrad et al., 2014). For volumetric assessments, individual brains were segmented into 22 different regions of interest (ROI) using the pig brain atlas. Total brain and individual region volume analysis was performed with SPM8 in which an inverse warp file for each ROI was generated from the DARTEL-generated warp files for each region. Generation of region-specific warp files was previously described (Radlowski et al., 2014, Mudd et al. (2016a)). In order to account for absolute

whole-brain volume, all regions of interest were also expressed as a percent of total brain volume (%TBV), using the following equation (within subject): $\left(\frac{ROI\ absolute\ volume}{total\ absolute\ volume}\right) * 100$.

5.3.4.2 Diffusion Tensor Imaging

Diffusion tensor imaging was used to assess white matter maturation and axonal tract integrity using a $b = 1000\ s/mm^2$ across 30 directions and a 2 mm isotropic voxel. Diffusion-weighted echoplanar images (EPI) were assessed in FSL 5.0 for fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD) using methods previously described (Mudd et al., 2016b). The pig brain atlas was used for assessment of the following regions of interest: caudate, corpus callosum, cerebellum, both hippocampi, internal capsule, left and right cortex, thalamus, DTI-generated white matter, and atlas-generated white using a customized pig analysis pipeline and the FSL software package. The pig brain atlas was used for analysis. The diffusion toolbox in FSL was used to generate values of AD, RD, MD, and FA. In the corresponding results, atlas-generated white matter indicates the use of white matter prior to using probability maps from the piglet brain atlas that were used as a region of interest mask. Likewise, DTI-generated white matter indicates a threshold of 0.2 was applied to FA values, thus restricting analysis to white matter tracts. Masks for each ROI from the atlas were non-linearly transformed into the MPRAGE space of each individual pig and a linear transform was then applied to transfer each ROI into DTI space. A threshold of 0.5 was applied to each ROI, and the data were dilated twice. For each individual ROI, an FA threshold of 0.15 was applied to ensure that we included only white matter in that region of interest despite the mask expansion.

5.3.4.3 Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy was used to non-invasively quantify metabolites in the whole brain. The MRS spin-echo chemical shift sequence was used with a voxel size of 20 mm x 23 mm x 13 mm and centered over the left and right dorsal hippocampi. The following sequence parameters were used in acquisition of spectroscopy data for the water-suppressed scan: TR = 1800 ms; TE = 68 ms; signal averages = 256, vector size= 1024. The following sequence parameters were used in acquisition of spectroscopy data for the non-water-suppressed scan: TR = 20000 ms; TE = 68 ms; signal averages = 1; vector size = 1024 point. Both water-suppressed and non-water-suppressed data were collected in institutional units, and all MRS data were analyzed with LC Model (version 6.3) using methods previously described (Radlowski et al., 2014). Limits were placed on MRS data for inclusion in the statistical analysis. Cramer-Rao lower bounds (i.e., % standard deviation) were calculated using LC Model and only metabolites with a standard deviation less than 20% were considered to have reliable quantitative results of absolute levels.

5.3.5 Hippocampal Gene Expression

Approximately 20 mg of hippocampal tissue were introduced in a Lysing Matrix D tube (MP Biomedicals, Santa Ana, California, USA), placed on ice, and 650µL of lysis buffer (Agencourt RNAdvance Tissue Kit, Beckman Coulter, Indianapolis, Indiana, USA) was added. Tubes were agitated for 2x1 minutes at speed 6 on FastPrep®-24 (MP Biomedicals, Santa Ana, California, USA), and 400 µL of lysate were then extracted using the Agencourt RNAdvance Tissue Kit (Beckman Coulter, Indianapolis, Indiana, USA) following the manufacturer's recommendations. RNA were quantified using the Quant-iT™ RiboGreen™ RNA Assay Kit (Invitrogen, Carlsbad, California, USA) on a Spectramax M2 (Molecular Devices, Sunnyvale, California, USA). RNA quality assessment was completed using a Fragment Analyzer 96 with Standard Sensitivity RNA Analysis Kit (15 nt) (Advanced Analytical Technologies, Inc., Ankeny, Iowa, USA). Relative mRNA copy number on 93 genes was quantified using the NanoString nCounter™ system (NanoString Technologies Inc., Seattle, Washington, USA) according to the manufacturer's instructions using 100 ng of RNA as the starting amount. Relative copy number was obtained by normalizing the absolute copy number of each gene samples to the geometric mean of all housekeeping genes.

5.3.6 Statistical Analysis

Data analysis was conducted using the GLIMMIX procedure of SAS Enterprise Guide 7.1 (SAS Institute, Cary, NC, USA). All data were subjected to a one-way analysis of variance to assess the effect of dietary treatment. Cohort of pigs was included in the model as a random variable. For all variables, observations with a studentized residual greater than |3| were considered outliers and removed from that variable only. For behavioral data, pigs that exhibited little exploration of either object (i.e., less than 2 seconds of exploration of the sample objects) were considered non-compliant and their recognition index was not measured in the test phase, but all other exploration measures were included for those subjects. Performance in the sample phases from the 1- and 48-hour delay paradigms were averaged to create pooled sample phase exploration measures. To test for recognition memory, a one-sample t-test was conducted comparing the recognition index to a null mean of 0.5. Groups with a mean recognition index significantly above 0.5 were considered to demonstrate recognition memory.

For individual brain region volume assessment, volume was expressed in both absolute (i.e., mm^3) and relative units (i.e., regional volume as a proportion of total brain volume, within subject). Gene expression data were standardized (mean of zero and standard deviation of one) and centered by the control group, thus all scores for the control group are zero. Statistical significance was defined at $p < 0.05$ (insignificant results not shown). Post-hoc comparisons for mean separation were conducted with a Tukey adjustment, and data are represented as least square means. Correlations between significant outcomes (MRI or gene expression) gene expression and the recognition index were conducted using the Pearson correlation coefficient for each diet and linear regression was used to assess the diet independent relationship between outcomes.

5.4 Results

5.4.1 Behavior

The control group failed to exhibit recognition memory after either 1- or 48-h delay. The OF group was able to show recognition memory after a 1-h delay (one-sample t-test, $p < 0.001$) but not after a 48-h delay (one-sample t-test, $p = 0.155$). On the other hand, the OF + 2'FL group failed to show recognition memory after 1-h delay (one-sample t-test, $p = 0.592$), but was able to show recognition after a 48-h delay (one-sample t-test, $p = 0.001$, Figure 5.1A). Exploratory behaviors (e.g., distance moved, time spent exploring objects, frequency of object visits, mean length of object visits) were similar between groups during both habituation trials, the sample trials, and the 48-h delay test trial (data not shown). After a 1-h delay, the OF group demonstrated more frequent visits to the novel object compared with the CON group ($p = 0.022$), whereas the control group maintained a high rate of exploration of the novel object throughout the trial ($p = 0.045$). On the contrary, exploration of the sample object by the OF + 2'FL group increased (pigs exhibited a positive rate of exploration (seconds exploring/minute)) as the trial went on compared to the CON group ($p = 0.038$, Figure 5.1).

5.4.2 MRI

A 3D surface rendering of the brain regions affected by the diet is shown in Figure 5.2. Trending effects of diet were observed for the absolute volumes of the olfactory bulbs, caudate, internal capsule, and thalamus, whereas the relative volumes of the caudate, cerebrospinal fluid, and both hippocampi exhibited a trend effect of the diet ($0.05 < p < 0.10$, Figure 5.3, Supplemental Tables 2-3). The only significant effect observed was an increase of the relative volume of the olfactory bulbs in the OF and OF + 2'FL groups compared with the CON group ($p = 0.019$, Figure 5.3). There was no effect of diet for any DTI measure (AD, MD, RD, or FA, all $p > 0.165$, Supplemental Tables 4-5). Of the MRS outcomes, only glutathione, myo-inositol, N-acetylaspartate, and γ-amino butyric acid met criteria for inclusion, however none were altered by diet (all $p > 0.303$, Supplemental Table 6).

5.4.3 Hippocampal Gene Expression

The OF and OF + 2'FL groups largely had opposite effects compared to the CON group. Hippocampal mRNA expression of the dopamine receptor D3 (*DRD3*), the GABA type B receptor subunit 1 (*GABBR1*), the histone deacetylases 5 and 8 (*HDAC5/8*), the neural cell adhesion molecule 1 (*NCAM1*), and the cholinergic receptor muscarinic 2 (*CHRM2*) were all downregulated in the OF group (all $p < 0.045$, Table 1, Figure 5.4A). Except for *HDAC8* and *CHRM2*, these same genes were upregulated in the OF + 2'FL group compared to controls (all $p < 0.045$). To visualize the general trend for mRNA to be up- or downregulated as compared to controls, within diet, genes were ordered by descending Z-score and plotted on a heatmap

(Figure 5.4B). All results can be found in Supplemental Table 7.

5.4.4 Correlation and Linear Regression

Linear regression (independent of diet) and correlations (by diet group) were conducted between only those variables significantly affected by the diet. Although collection of hippocampal tissue, MRI, and behavioral data were collected on separate days, there were significant study-wide relationships between the recognition index after a 1-h delay and *CHRM2* (β_1 = -0.08, $p = 0.01$), *GABBR1* (β_1 = -0.13, $p < 0.01$), and *HDAC5* (β_1 = -0.11, $p < 0.01$) expression. When correlations were assessed by diet, these relationships appeared to be driven by specific dietary groups (Figure 5.5, Supplemental Table 8). No relationships were observed between gene expression data and the recognition index after a 48-hour delay (β_1, all $p > 0.10$).

5.5 Discussion

The objective of this study was to assess the effectiveness of OF alone or in combination with 2'FL at altering recognition memory, brain structure, and hippocampal gene expression. Dietary intake of OF alone produced effects differential to that of OF + 2'FL concerning recognition memory and hippocampal gene expression; however both increased the relative volume of the olfactory bulbs compared to controls. This study adds to a growing body of literature suggesting OS are capable of altering behavior and neurobiology, however the mechanisms of action remain unclear. While many OS have been cited as having such effects (Kao et al., 2016), it is becoming clear that not all OS act similarly or with the same efficacy.

We chose to use the NOR task using two different delays to assess intermediate (1-h delay) and long-term (48-h delay) object recognition memory. While CON pigs failed to exhibit recognition memory after both the 1- and 48-h delays, pigs fed OF exhibited recognition memory after a 1-h delay, whereas pigs fed OF + 2'FL exhibited recognition memory after a 48-h delay. Pigs fed OF displayed greater number of visits and quicker habituation to the novel object after a 1-h delay, whereas those fed OF + 2'FL did not habituate to the sample object, but rather maintained a high rate of exploration throughout the trial. Aside from recognition memory, all groups behaved similarly (e.g., total distance moved, frequency, total duration, and mean length of object visits) after a 48-h delay. Though it may appear concerning that the control group was unable to complete the task, we have previously reported a similar phenomenon wherein pigs fed a diet without prebiotics (polydextrose and galactooligosaccharide) could not demonstrate recognition memory (Fleming et al., 2017). In a follow-up study where the control diet was then supplemented with those same prebiotics and the test diet supplemented further with sialyllactose, no differences in behavioral performance were observed (Fleming et al., 2018). Given recognition memory is measured behaviorally in a binary manner (presence or absence thereof), if the goal is to demonstrate a cognitive promoting effect of a nutrient, the use of a control group that is unable to complete the task is necessary to detect subtle improvements in recognition memory.

Where diet had a significant impact on behavioral outcomes, it had no impact on MRS or DTI outcomes, and of the 22 brain regions investigated, only the relative size of the olfactory bulbs was affected. Interestingly, in humans aged 1-17 years of age, absolute volume of the olfactory bulb increases with age whereas the relative volume decreases continuously starting the first year of age (Hummel et al., 2011). Olfactory bulb function was correlated with olfactory bulb volume, with increasing volume correlated with increasing function (Hummel et al., 2011, Buschhüter et al. (2008)). Notably, the volume of both olfactory bulbs together in the neonatal pig is approximately over 13 times larger than the peak volume of olfactory bulbs in humans, which occurs near 40 years of age (~2200 mm^3 vs ~160mm^3; values adapted from Buschhuter (Buschhüter et al., 2008)). As function and volume are related, comparisons between species whose olfactory bulbs differ to such a great extent are difficult. Animals studies have found that olfactory deprivation results in reduction of olfactory bulb size in opposums (Cummings et al., 1997) and vascular density in rats (Korol and Brunjes, 1992), demonstrating a strong link between size and function. We found no relationship between recognition memory and relative olfactory bulb volume (data not shown), however a true test of olfactory function would be required to relate olfactory size and function in the pig. Furthermore, objects used in the NOR task were cleaned with detergent containing water to reduce the influence of odor cues on recognition of the familiar object, thus it may be appropriate that a link between recognition memory and olfactory bulb volume was not observed. In context of nutritional intervention, this is the third time we have found a link between early life diet and olfactory bulb anatomy. We had previously found that the olfactory bulbs experience compensatory growth in pigs provided iron-replete diets after a period of neonatal dietary iron deficiency (Mudd et al., 2018b), and that despite loss of iron in many brain regions (e.g., pons, medulla, cerebellum, left cortex, and left hippocampus), the olfactory bulb was unique in retaining iron (Mudd et al., 2018a). Together with the present data demonstrating an increase in relative volume of the olfactory bulb after OS supplementation, these data suggest the pig olfactory bulbs may be preferentially protected or promoted in context of nutritional deficiency or supplementation.

Although the change in relative volume of the olfactory bulb was the only statistically significant outcome, a statistical trend for a change in volume of several brain regions warrants investigation. It is notable that all brain regions affected by the diet were subcortical. The absolute volumes of the caudate, internal capsule, and thalamus and relative volumes of the caudate, cerebrospinal fluid, and both hippocampi were sensitive to diet. Although volumes (absolute or relative) of the olfactory bulbs, caudate, and internal capsule are similar between OF and OF + 2'FL groups, in the latter group, there was a trend for the relative volumes of the left and right hippocampi to be smaller compared to CON and OF groups. This further supports the emerging and consistent pattern where several measures (behavior, structural, or genetic) were divergently affected between OF and OF + 2'FL groups. Hippocampal function has been traditionally associated with behavioral tasks requiring integration of spatial cues or retention of information over a long period of time. In regard to the novel object recognition task, a study in mice has shown that hippocampal lesion only impaired novel object recognition with a delay of 24-h but not with a delay of 5-min (Hammond et al.,

2004). It is only when recognition memory contains a spatial component (such as the context or location of a stimulus) or long delay that the hippocampus is required, otherwise recognition of "what" was seen requires the perirhinal cortex (Barker and Warburton, 2011). It is therefore surprising to observe in the OF + 2'FL group a trend toward a reduction of relative hippocampi volume ($0.05 < p < 0.1$) concomitant with an increased performance in the novel object recognition with a long delay (48-h). Here, the absolute volumes of the hippocampi were similar between groups, however the relative decrease in volume in the OF + 2'FL group may suggest a shift in the process of synaptogenesis and/or myelination. Conversely, given the stability between groups in absolute volume, it is possible that the reduction in relative volume of the hippocampi is an artifact of more significant growth in other brain regions.

While this study confirms several reports showing various OS improve behavioral performance in both human and animal models (Yen et al., 2017, Vázquez et al. (2015), Vazquez et al. (2016), Fleming et al. (2017), Schmidt et al. (2015), Jia et al. (2016), Savignac et al. (2016)), it is one of the first to examine the potential of human and non-human milk OS together. Adult mice and rats fed chow containing 0.312% or 0.625% 2'FL for 12 or 5 weeks, respectively, showed increased and longer lasting potentiation of Schaffer collateral neurons in the CA1 region of the hippocampus (Vázquez et al., 2015). Supplemented mice displayed increased performance on place learning, working memory, and fixed-ratio lever-pressing tasks in an operant box, suggesting 2'FL supplementation enhanced multiple cognitive domains. Expression of the postsynaptic density 95 (PSD-95) protein in supplemented rats qualitatively appeared increased upon immunohistochemical analysis in the hippocampus, frontal cortex, and striatum, which was confirmed by Western blot analyses in the hippocampus and frontal cortex. Additionally, calcium/calmodulin-dependent protein kinase II (CaMKII), a protein involved in long-term potentiation, was increased in the hippocampus, and BDNF was elevated in both the hippocampus and striatum (Vázquez et al., 2015). Similarly, oral gavage with 3 g/kg FOS or 4 g/kg GOS for 5 weeks has been shown to differentially alter BDNF, NMDAR, and plasma D-serine in adult male rats (Savignac et al., 2013). BDNF and the glutamatergic NMDA receptor NR1 were greater in the hippocampus of those fed FOS, whereas NR1 was greater in the frontal cortex and NR2 greater in the hippocampus of those fed GOS. Interestingly, we did not see an increase in mRNA expression of *BDNF*, any of the glutamate ionotropic receptor NMDA type subunits (*GRIN1*, *GRIN2A/B/C/D*), or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (*AMPA*) type subunits (*GRIA1-4*). We noted similar departures from rodent work in a previous study (Fleming et al., 2017), and these differences may be accounted for by a myriad of differences such as outcome (protein or mRNA expression), animal model (pig or rodent), and the relative difference in development over the course of a four week period between pigs and rodents.

Of the genes affected by diet in the present study, it appeared that pigs fed OF displayed opposite effects of those fed OF + 2'FL, this is evident in the pattern shown in Figure 5.4B. Overall, pigs fed OF demonstrated greater hippocampal downregulation as compared to controls than pigs fed OF + 2'FL. Specifically, pigs

fed OF displayed reduced gene expression of *DRD3*, *GABBR1*, *HDAC5/8*, *NCAM1*, and *CHRM2* relative to controls. Pigs fed OF + 2'FL displayed increases of all of the previous genes except for *HDAC8* and *CHRM2*. Although the magnitude of expression was similar for *CHRM2*, a gene known to be related to cognition in humans (Gosso et al., 2007), the downregulation of *HDAC5* was greater for pigs fed OF than those fed OF + 2'FL. Such a pattern may be related to the apparent difference in behavior observed in the NOR task, but it remains difficult to reconcile the differential effects of OF and OF + 2'FL on gene expression given their apparent benefit to recognition memory. Of the affected genes, *CHRM2*, *GABBR1*, and *HDAC5* were inversely correlated with recognition index after a 1-h delay. While significant relationships were observed overall, these appeared to be driven by specific dietary groups. The relationship between the recognition index and *CHRM2* was strongest in the OF + 2'FL group, whereas the relationship between *HDAC5* and recognition index was driven by the CON and OF + 2'FL group. All dietary groups showed an inverse relationship between *GABBR1* and the recognition index, and linear regression revealed the strongest and most significant relationship between these two outcomes. None of the affected genes were related to the recognition index after a 48-h delay, suggesting a different mechanism for the behavioral demonstration of recognition memory after intermediate and long delays, and that these mechanisms are sensitive and differentially altered by oligosaccharide supplementation. The idea that genes are differentially expressed in context of time or familiarity/novelty of a stimuli is not new (Cavallaro et al., 2002, Scott et al. (2017)), however the possibility that dietary oligosaccharides may differentially alter such processes is novel. The divergence in performance on the NOR task and *GABBR1* expression (and many other genes) by the OF and OF + 2'FL groups together with the relationship between the recognition index and *GABBR1* expression highlight a potential mechanistic link connecting the two phenomena.

The discovery that prebiotics improve cognition is relatively recent, and to our knowledge there has been little investigation into the connection between prebiotic intake and GABAergic processes. Some information may be gleaned from recent work with probiotics. BALB/c mice orally gavaged with broth containing *L. rhamnosus* (JB-1) for 28 d displayed greater movement in an open field test, less time immobile during a forced swim test, greater entries to the open arm in an elevated plus maze, and increased memory on a fear conditioning task; indices demonstrating reduced response to stress and improved memory (Bravo et al., 2011). These changes were concomitant with reduced mRNA expression of the $GABAB1_b$ receptor in the amygdala, locus coeruleus, and hippocampus, and increased expression in the cingulate 1 and prelimbic cortices. A follow-up study using magnetic resonance spectroscopy in mice provided JB-1 demonstrated increased brain GABA after 4-weeks of consumption (Janik et al., 2016).

In context of object recognition, it appears that hippocampal reductions in GABAB receptor expression are beneficial. Cavallaro et al. (Cavallaro et al., 2002) proposed that downregulation of hippocampal GABAB receptor signaling may improve short term memory measured by the Morris water maze upon review of whole-genome screening. In support of this hypothesis, Baclofen, a GABAB receptor agonist, has

been shown to dose-dependently impair acquisition and storage of object recognition memory, whereas the GABAB receptor antagonists CGP 35348 (P-(3-aminopropyl)-P-diethoxymethylphosphinic acid) and nitric oxide donor, molsidomine, prevent baclofen-induced impairments (Pitsikas et al., 2003). Although the effects of GABAB agonism and antagonism vary by dose, route, and behavioral task used (for review see Heaney and Kinney (Heaney and Kinney, 2016)), it appears that both oligosaccharides and probiotics (Bravo et al., 2011, Janik et al. (2016)) are linked to beneficial alteration of GABA receptor expression in the brain.

Many potential links have been proposed as mechanisms within the gut-brain-axis, which include circulatory transport of hormones (Mudd et al., 2017a) or volatile fatty acids (VFA, such as acetate, propionate, or butyrate) (Stilling et al., 2016), modulation of the immune system (Sampson and Mazmanian, 2015), and vagal communication (Vazquez et al., 2016, Bravo et al. (2011)). Volatile fatty acids have been proposed to be strong modulators of the gut-brain-axis due to their HDAC inhibitor activity (Bourassa et al., 2016), regulation of satiety (Frost et al., 2014), stimulation of neurogenesis (Kim et al., 2009), and regulation of the blood-brain-barrier (Braniste et al., 2014). We found that mRNA expression of HDAC8 was decreased in both groups fed OF, however expression of *HDAC5* was decreased in pigs fed OF and increased in pigs fed OF + 2'FL. Histone deacetylases regulate the transcription of many genes, therefore the effect of the diet on these regulators may be responsible for the downstream effects on the other genes measured. Importantly, non-specific HDAC inhibition via sodium butyrate has been shown to modulate long-term object recognition memory in mice (Stefanko et al., 2009). VFA are HDAC inhibitors, and we observed that decreases in *HDAC5* mRNA are correlated with increases in the recognition index; we therefore hypothesize that fermentation of OF and/or 2'FL and production of VFA may indirectly impact recognition memory via alteration of HDAC expression. In addition to VFA, recent research suggests the vagus nerve may be a linchpin between gut-brain communication and cognition. Bravo et al. (Bravo et al., 2011) demonstrated that intake of *L. rhamnosus* did not alter performance on the open field, forced swim test, or expression of GABAB receptors after bilateral subdiaphragmatic vagotomy. In a separate study subdiaphragmatic bilateral vagotomy was used to assess the requirement of the vagus nerve for 2'FL mediated increases in cognition in rodents (Vazquez et al., 2016). Vagotomy abolished 2'FL mediated increases in hippocampal long-term potentiation, however all groups (sham/vagotomy and control/2'FL) were still able to perform above criterion in a fixed-ratio lever pressing task. By the end of training, 2'FL/sham animals displayed greater lever presses than 2'FL/vagotomy or control/vagotomy animals, indicating that while not required for behavioral performance, vagal communication is necessary for 2'FL induced increases. Together, these data suggest that the vagus nerve has a crucial role in mediating gut-brain related increases in cognitive ability.

5.6 Conclusion

We investigated the impact of OF alone or in combination with 2'FL on cognition, structural brain development, and hippocampal gene expression. Feeding either OS had little impact on brain structure, however they had differential effects on cognition and hippocampal gene expression. These data highlight the potential for OS to be used to promote cognition however more research on the underlying mechanisms is warranted.

5.7 Table

Table 5.1: mRNA expression.[*†]

Measure	CON	OF	OF + 2'FL	Pooled SEM	p-value
$CHRM2$[‡]	0	-0.75	-0.77	0.32	0.045
$DRD3$	0^{ab}	-0.47^{b}	0.67^{a}	0.26	0.016
$GABBR1$	0^{ab}	-0.52^{b}	0.42^{a}	0.32	0.037
$HDAC5$	0^{ab}	-0.45^{b}	0.62^{a}	0.30	0.012
$HDAC8$	0^{a}	-1.27^{b}	-0.35^{a}	0.25	0.003
$NCAM1$	0^{ab}	-0.74^{b}	0.35^{a}	0.29	0.011

[*] Abbreviations: CON, control group; OF, pigs fed oligofructose; OF + 2'FL, pigs fed oligofructose and 2'fucosyllactose; SEM, standard error of the mean; $CHRM2$, cholinergic receptor muscarinic 2; $DRD3$, dopamine receptor D3; $GABBR1$, GABA type B receptor subunit 1; $HDAC5/8$, histone deacetylases 5 and 8; $NCAM1$, neural cell adhesion molecule 1

[†] Standardized values for mRNA expression (mean = 0, standard deviation = 1) centered by control group. Only measures significantly altered by diet are shown. Data were analyzed via two-way ANOVA with a post-hot Tukey adjustment for multiple comparisons. Means without a common superscipt differ ($p < 0.05$)

[‡] Mean separation insignificant after Tukey adjustment

5.8 Figures

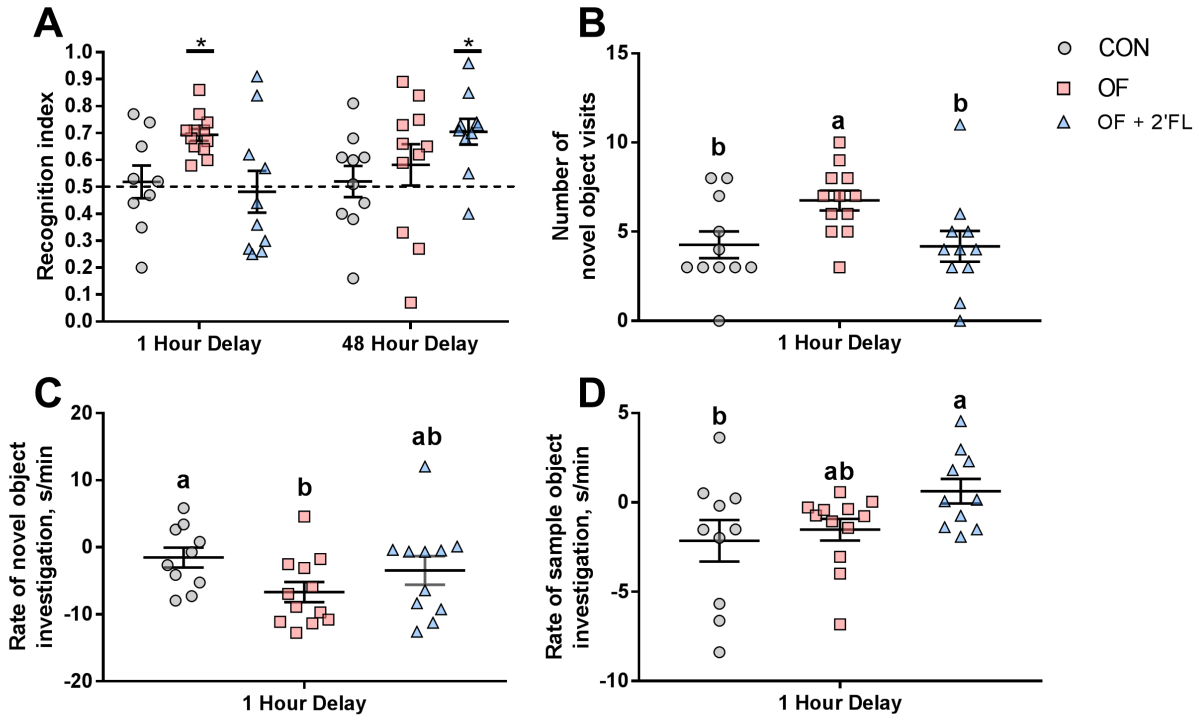

Figure 5.1: Recognition memory and exploratory behavior during the novel object recognition task. **A)** The OF group was able to show recognition memory after a 1-h delay (one-sample t-test, $p < 0.001$), however only the OF + 2'FL group was able to show recognition memory after a 48-h delay (one-sample t-test, $p = 0.001$). Exploratory behavior was similar between groups after a 48-h delay, however differences emerged after a 1-h delay. **B)** The OF group visited the novel object more frequently than the CON group ($p = 0.022$), **C)** whereas the control group maintained a high rate of exploration of the novel object throughout the trial ($p = 0.045$). That is, the OF group habituated to the novel object more quickly than the CON group. **D)** On the contrary, exploration of the sample object by the OF + 2'FL group increased as the trial went on compared to the CON group ($p = 0.038$). Lines depict mean + standard error, groups without a common superscript differ ($p < 0.05$). Abbreviations: CON, control group; OF, pigs fed oligofructose; OF + 2'FL, pigs fed oligofructose and 2'fucosyllactose.

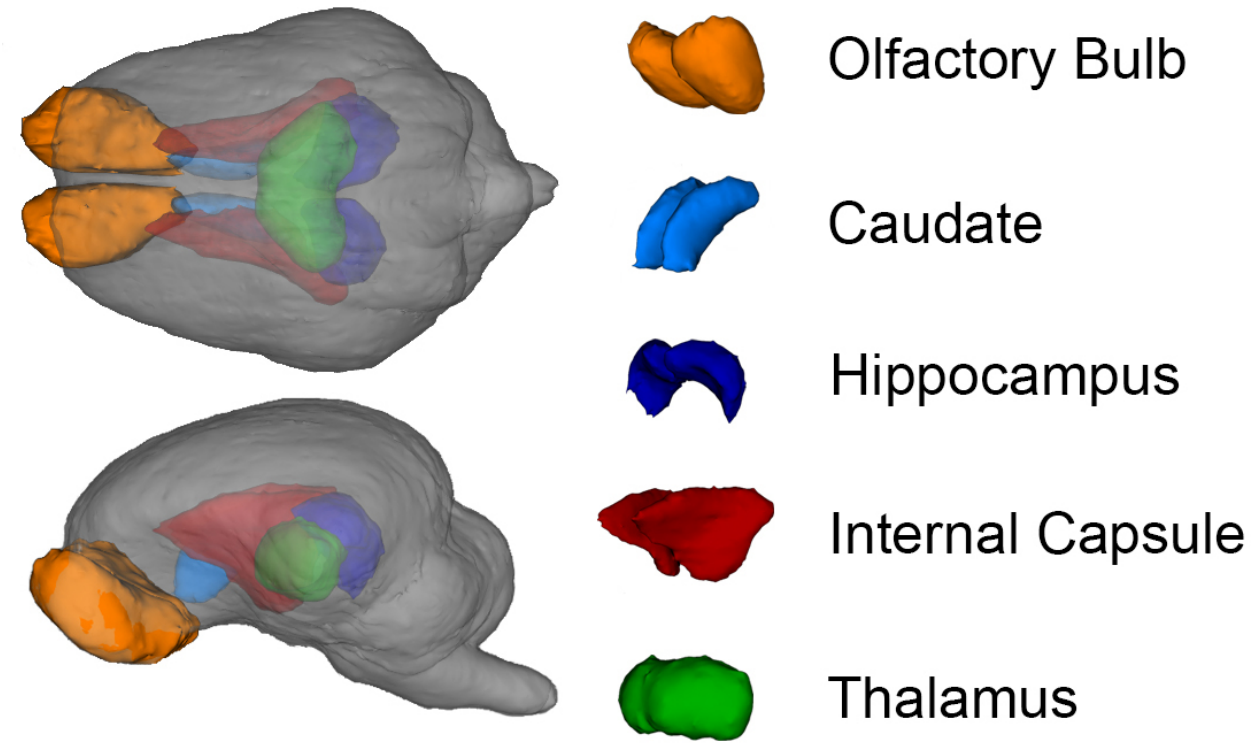

Figure 5.2: Representative 3D surface rendering of the brain from the pig brain atlas highlighting regions affected by diet.

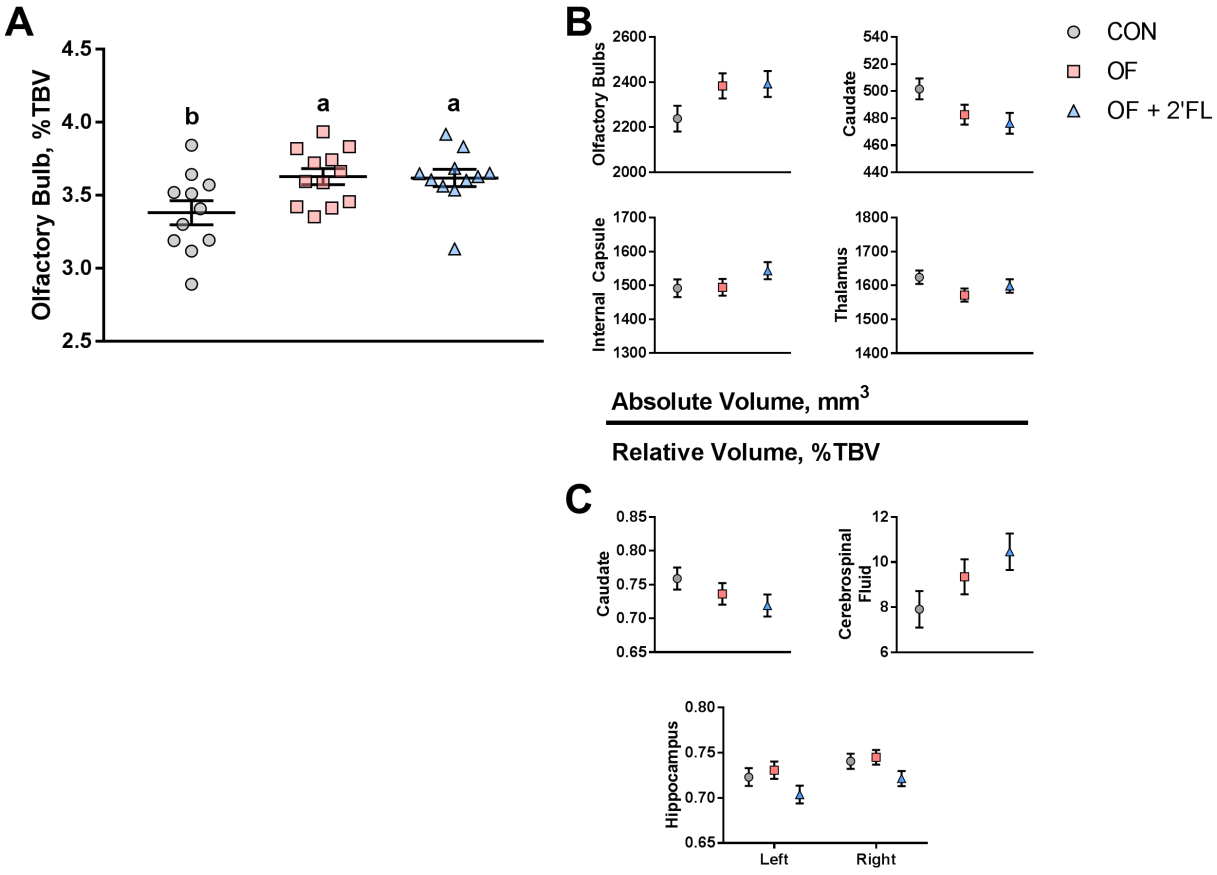

Figure 5.3: **A)** Both the OF and OF + 2'FL groups demonstrated larger relative volumes of the olfactory bulbs ($p = 0.019$) as compared to controls. **B)** Trending effects of diet are shown for both absolute and **C)** relative brain volumes ($0.05 < p < 0.10$). Lines depict mean/symbol + standard error, groups without a common superscript differ ($p < 0.05$). Abbreviations: CON, control group; OF, pigs fed oligofructose; OF + 2'FL, pigs fed oligofructose and 2'fucosyllactose; %TBV, percent of total brain volume.

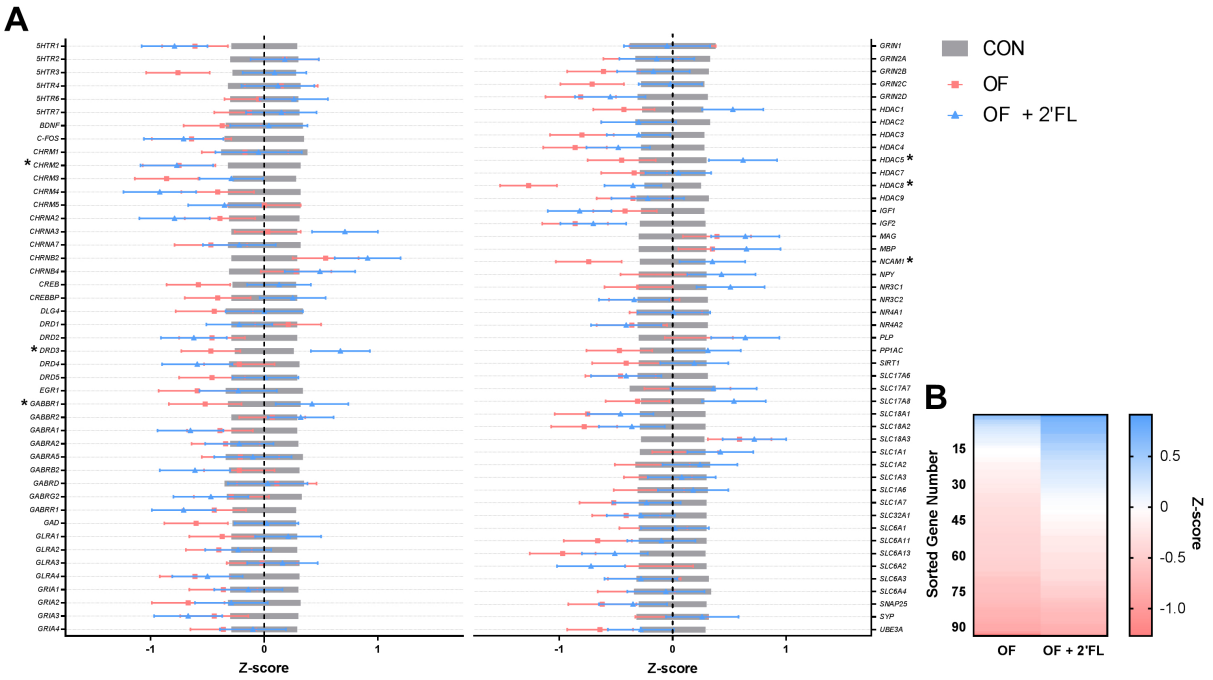

Figure 5.4: Hippocampal tissue was assessed for the mRNA expression of 93 genes. **A)** Figure depicts standardized data (mean = 0, standard deviation = 1) centered by control group. Values below zero indicate decreased expression compared to control, whereas values above zero indicate increased expression. Bars show mean + standard error, genes significantly impacted by diet are denoted by an asterisk. **B)** Genes were sorted in descending order by Z-score for each diet, visualizing the abundance of downregulated gene products in the OF group compared to the OF + 2'FL group Abbreviations: CON, control group; OF, Pigs fed oligofructose; OF + 2'FL, pigs fed oligofructose and 2'fucosyllactose.

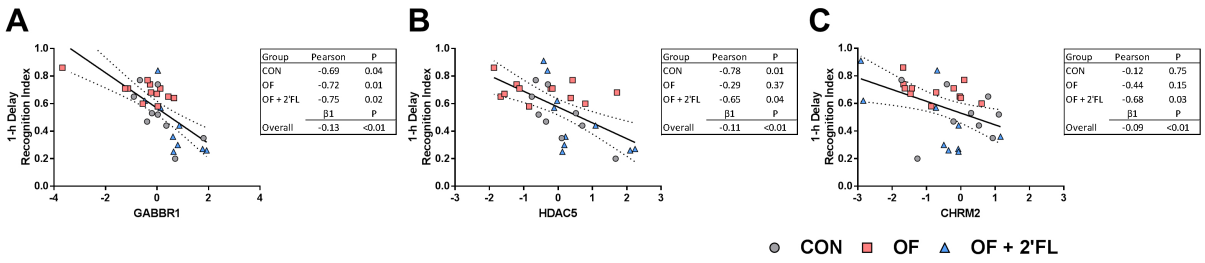

Figure 5.5: Significant correlations by diet or study-wide linear regression between the recognition index after a 1-h delay and genes affected by diet. No significant relationships were found with the recognition index after a 48-h delay. **A)** A negative correlation for all diets was found between *GABBR1* and the recognition index. Linear regression demonstrated the presence of a significant relationship, for every increase in 1 standard deviation of *GABBR1* expression, the recognition index decreased by 0.13. **B-C)** Some dietary groups showed a correlation between the recognition index and either *HDAC5* or *CHRM2*. Overall, linear regression revealed significant negative relationships between mRNA expression and the recognition index. Abbreviations: *GABBR1*, GABA type B receptor subunit 1; *HDAC5*, histone deacetylase 5; *CHRM2*, cholinergic receptor muscarinic 2; CON, control group; OF, pigs fed oligofructose; OF + 2'FL, pigs fed oligofructose and 2'fucosyllactose.

Chapter 6

Human and bovine milk oligosaccharides elicit improved recognition memory concurrent with alterations in regional brain volumes and hippocampal mRNA expression[1]

6.1 Abstract

Human milk contains a unique profile of oligosaccharides (OS) and preliminary evidence suggests they impact brain development. The objective of this study was to assess the impact of bovine and/or human milk oligosaccharides (2'fucosyllactose [2'FL] and Lacto-N-neotetraose [LNnT]) on cognition, brain development, and hippocampal gene expression. Beginning on postnatal day (PND) 2, male pigs received one of four milk replacers containing bovine milk oligosaccharides (BMOS), human milk OS (HMO), both (BMOS + HMO), or neither (CON). Pigs were tested on the novel object recognition task using delays of 1- or 48-h at PND 22. At PND 32-33, magnetic resonance imaging (MRI) procedures were used to assess structural brain development and hippocampal tissue was collected for analysis of mRNA expression. Pigs consuming only HMO exhibited recognition memory after a 1-h delay and those consuming BMOS + HMO exhibited recognition memory after a 48-h delay. Both absolute and relative volumes of cortical and subcortical brain

[1]Human and bovine milk oligosaccharides elicit improved recognition memory concurrent with alterations in regional brain volumes and hippocampal mRNA expression, *Scientific Reports*. Under review.

regions were altered by diet. Hippocampal mRNA expression of *GABRB2*, *SLC1A7*, *CHRM3*, and *GLRA4* were most strongly affected by diet. HMO and BMOS had distinct effects on brain structure and cognitive performance. These data suggest different mechanisms underly their influence on brain development.

6.2 Introduction

Human milk oligosaccharides (HMO) are increasingly being recognized as important promoters of intestinal and immune development (Kirmiz et al., 2018). Human milk oligosaccharides are the third most concentrated solid in human milk behind lactose and lipids (Bode, 2012), and their diversity and concentration are unlike any other species (Urashima et al., 2001). Although bovine milk contains OS that are structurally similar or identical to those found in human milk, they are found in lower concentrations than in human milk (Gopal and Gill, 2000, Martín-Sosa et al. (2003)). Emerging research has suggested that bovine milk derived oligosaccharides (BMOS) and HMO (e.g., 2'fucosyllactose (2'FL), Lacto-N-neotetraose (LNnT)) may confer physiological benefits when consumed by formula-fed infants.

Infants fed formula supplemented with BMOS have shown tolerance and adequate growth as measured by anthropomorphic measures (e.g., weight-for-age, length-for-age, or head-circumference scores) and stool characteristic (e.g., frequency, consistency, or pH) closer to that of breastfed infants (Meli et al., 2014, Cooper et al. (2016), Radke et al. (2017), Simeoni et al. (2016)). Provision of a combination of BMOS and *Bifidobacterium animalis subsp. Lactis* brought fecal *bifidobacteria* counts of infants delivered via C-section closer to that of infants delivered vaginally (Cooper et al., 2016), and the same combination brought 16S ribosomal RNA sequencing indices of richness, evenness, and the Shannon index similar to that of a breastfed control group.

Infants fed formula supplemented with HMO demonstrated age appropriate growth (Puccio et al., 2017, Marriage et al. (2015)), fewer reports of respiratory illness or use of antibiotics/antipyretics (Puccio et al., 2017), and stool consistency and frequency closer to that of breastfed infants (Marriage et al., 2015). Absorption and excretion of 2'FL is consistent with the amount of 2'FL consumed, with breastfed and 2'FL supplemented infants displaying higher concentrations of plasma and urinary 2'FL (Marriage et al., 2015). Furthermore, infants fed formula containing 2'FL in addition to galactooligosaccharide (GOS) demonstrated plasma cytokine (e.g., IL-1ra, TNF-α, IL-1α, IL-1β, IL-6) concentrations closer to that of breastfed infants than those consuming GOS only supplemented formulas. When peripheral blood mononuclear cells were stimulated with respiratory syncytial virus ex vivo the cytokine response from breastfed and 2'FL supplemented infants were similar (Goehring et al., 2016).

Recent reports demonstrate that various oligosaccharides are also capable of promoting brain development (Kao et al., 2016). Rats supplemented with 2'FL demonstrated vagally mediated improved learning and memory (Vázquez et al., 2015, Vazquez et al. (2016), Oliveros et al. (2016)), rats supplemented

with sialyllactose demonstrated improved behavioral response to stress (Tarr et al., 2015), and we have demonstrated that pigs fed a combination of polydextrose and galactooligosaccharide (Fleming et al., 2017) or 2'FL and oligofructose have improved performance in the novel object recognition task (Chapter 5). Various oligosaccharides have separately proven capable of altering brain or cognitive development in some manner, however few studies have directly compared the efficacy of BMOS and HMO.

Therefore, the objective of the present study was to assess the efficacy of BMOS and HMO alone or in combination at altering performance in the novel object recognition task, structural neurodevelopment, or hippocampal mRNA expression in the young pig.

6.3 Methods

6.3.1 Animals and housing

All animal care and experimental procedures were in accordance with the National Research Council Guide for Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. Forty-eight intact male pigs (1050 Cambro genetics) were naturally farrowed and allowed colostrum consumption for up to 48 h before transport to the Piglet Nutrition & Cognition Laboratory at the University of Illinois at Urbana-Champaign. Pigs were artificially reared from postnatal day (PND) 2 until PND 33. This study was conducted using six independent cohorts (n = 2 pigs per dietary treatment in each cohort), with pigs selected to control for genetics and initial body weight within cohort. All pigs were housed in master caging units that contained six individual stainless-steel cages (L × W × H of 87.6 × 88.9 × 50.8 cm) with clear, polycarbonate facades on three sides of the cage and vinyl-coated, expanded-metal flooring (Tenderfoot®, Minneapolis, MN). The master unit was designed such that there were three separate levels each with two individual pig cages on each level. Pigs on each level shared a common wall containing holes to permit pigs to see, smell, hear, and minimally touch one-another. A towel and toy were included in each cage to provide enrichment, all pigs were removed from cages and allowed to socialize with each other for approximately 30 min each day, and all pigs were allowed *ad libitum* access to water at all times.

All pigs were reared in the same room with ambient temperature maintained between 27°C and 29°C and a 12-h light/dark cycle maintained from 0600 to 1800 h. Prior to placement in the artificial rearing system, pigs were administered 5.0 mL of *Clostridium perfingens* antitoxin C + D per the manufacturer's recommendations (Colorado Serum Company, Denver, CO, USA). At study conclusion (PND 33), pigs were anesthetized using a telazol:ketamine:xylazine solution (50.0 mg tiletamine plus 50.0 mg of zolazepam reconstituted with 2.50 mL ketamine [100 g/L] and 2.50 mL xylazine [100 g/L]; Fort Dodge Animal Health) by intramuscular injection at 0.03 mL/kg bodyweight. After anesthetic induction, pigs were euthanized via intracardiac administration of sodium pentobarbital (86.0 mg/kg of body weight; Euthasol, Virbac Animal Health, Fort Worth, TX).

Two pigs were removed from study due to failure-to-thrive (diet: BMOS + HMO).

6.3.2 Dietary Treatments

All researchers involved with conducting the study and acquiring and analyzing study results remained blind to dietary treatment identity until final data analyses were complete. Pigs (n = 12 per diet) were provided milk replacers reconstituted at 200 g of dry powder per 800 g of water. Reconstituted diets were formulated to contain 0 g/L milk OS (control [CON], ProNurse® Specialty Milk Replacer, Purina Animal Nutrition, Gray Summit, MO), 6.5 g/L BMOS (BMOS, Nestlé Product & Technology center, Konolfingen, Switzerland), 1.5 g/L HMO (HMO, 1.0 g/L of 2'FL + 0.5 g/L of LNnT, Glycom, Hørsholm, Denmark), or both bovine and human milk oligosaccharides (BMOS + HMO; 6.5 g/L of BMOS+1.0 g/L of 2'FL + 0.5 g/L of LNnT). Briefly, the BMOS product was derived from bovine whey and composed primarily of galactooligosaccharide and trace amounts of 3' and 6' sialyllactose and is the same as that used in previous clinical trials (Meli et al., 2014, Cooper et al. (2016), Radke et al. (2017)). All diets were formulated to contain supplemental lactose to balance the amount carbohydrate between diets. Post-hoc analysis of the oligosaccharide content of the formulated diets is shown in Table 6.1.

Pigs received small volumes (approximately 500 mL) of experimental diets on the day of arrival to the rearing facility to allow for adjustment to the milk replacer prior to the standard feeding regimen. Pigs were fed at a rate of 285 mL and 325 mL of reconstituted diet per kg bodyweight from PND 2-6 and PND 7-33, respectively. Individual pig bodyweight was recorded daily to determine the volume of milk to be dispensed to individual animals throughout the day. Meals were administered 10 times a day, approximately every 100 min, between 1100 and 0400 h using an automated feeding system.

6.3.3 Behavior

Pigs were tested on the novel object recognition (NOR) task using two different delays to assess intermediate and long-term recognition memory. Testing consisted of a habituation phase, a sample phase, and a test phase. During the habituation phase, each pig was placed in an empty testing arena for 10 min each day for two days leading up to the sample phase. In the sample phase, the pig was placed in the arena containing two identical objects and given 5 min for exploration. After a delay of 1- or 48-h the pig was returned to the arena for the test phase of the NOR task. During the test phase, the pig was placed in the arena containing one object from the sample phase and a novel object and allowed to explore for 5 min. Between trials, objects were removed, immersed in hot water with detergent, and rubbed with a towel to mitigate odor and the arena was sprayed with water to remove urine and feces. Objects chosen had a range of characteristics (i.e., color, texture, shape, and size), however the novel and sample objects only differed in shape and size. Only objects previously shown to elicit a null preference were used for testing (Fleming and Dilger, 2017). Task order was counterbalanced between replicates. Habituation trials began at PND 22 and testing on the

sample phase began on PND 24. Recognition index, or the proportion of time spent with the novel object compared to total exploration of both objects, was used to measure recognition memory. A recognition index significantly above 0.50 demonstrates a novelty preference and thus recognition memory.

6.3.4 Magnetic Resonance Imaging

All pigs underwent MRI procedures at PND 32 or 33 at the Beckman Institute for Advanced Science and Technology Biomedical Imaging Center using Siemens MAGNETOM Trio 3T equipment with a Siemens 32-channel head coil. Each pig underwent imaging protocols only once, and scans for each cohort of pigs were completed all on the same day. The pig neuroimaging protocol included three magnetization prepared rapid gradient-echo (MPRAGE) sequences and diffusion tensor imaging (DTI) to assess brain macrostructure and microstructure, respectively, as well as magnetic resonance spectroscopy (MRS) to obtain brain metabolite concentrations. In preparation for MRI procedures, anesthesia was induced using an intramuscular injection of telazol (50.0 mg of tiletamine plus 50.0 mg of zolazepam reconstituted with 5.0 DI water; Zoetis, Florham Park, NJ) administered at 0.07 mL/kg bodyweight, and maintained with inhalation of isoflurane (98% O_2, 2% isoflurane). Pigs were immobilized during all MRI procedures. Visual observation of each pig's well-being, as well as observations of heart rate, PO_2 and percent of isoflurane were recorded every 5 min during the procedure and every 10 min post-procedure until animals recovered. Total scan time for each pig was approximately 60 min. Imaging techniques are briefly described below.

6.3.4.1 Structural MRI

A T_1-weighted magnetization-prepared rapid gradient echo (MPRAGE) sequence was used to obtain anatomic images of the pig brain with a 0.7 mm isotropic voxel size. Three repetitions were acquired and averaged using SPM8 in Matlab 8.3, and brains were manually extracted using FMRIB Software Library (FSL) (FMRIB Centre, Oxford, UK). The following sequence specific parameters were used to acquire T_1-weighted MPRAGE data: repetition time (TR) = 1900 ms; echo time (TE) = 2.49 ms; 224 slices; field of view (FOV) = 180 mm; flip angle = 9°. Methods for MPRAGE averaging and manual brain extraction were previously described (Mudd et al., 2016b). All data generated used a publicly-available population-averaged pig brain atlas (http://pigmri.illinois.edu)(Conrad et al., 2014). For volumetric assessments, individual brains were segmented into 22 different regions of interest (ROI) using the pig brain atlas. Total brain and individual region volume analysis was performed with SPM8 in which an inverse warp file for each ROI was generated from the DARTEL-generated warp files for each region. Generation of region-specific warp files was previously described (Radlowski et al., 2014, Mudd et al. (2016a)). In order to account for absolute whole-brain volume, all regions of interest were also expressed as a percent of total brain volume (%TBV), using the following equation (within subject): $\left(\frac{ROI\ absolute\ volume}{total\ absolute\ volume}\right) * 100$.

6.3.4.2 Diffusion Tensor Imaging

Diffusion tensor imaging was used to assess white matter maturation and axonal tract integrity using a $b = 1000\ s/mm^2$ across 30 directions and a 2 mm isotropic voxel. Diffusion-weighted echoplanar images (EPI) were assessed in FSL 5.0 for fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD) using methods previously described (Mudd et al., 2016b). The pig brain atlas was used for assessment of the following regions of interest: caudate, corpus callosum, cerebellum, both hippocampi, internal capsule, left and right cortex, thalamus, DTI-generated white matter, and atlas-generated white using a customized pig analysis pipeline and the FSL software package. The pig brain atlas was used for analysis. The diffusion toolbox in FSL was used to generate values of AD, RD, MD, and FA. In the corresponding results, atlas-generated white matter indicates the use of white matter prior to using probability maps from the piglet brain atlas that were used as a region of interest mask. Likewise, DTI-generated white matter indicates a threshold of 0.2 was applied to FA values, thus restricting analysis to white matter tracts. Masks for each ROI from the atlas were non-linearly transformed into the MPRAGE space of each individual pig and a linear transform was then applied to transfer each ROI into DTI space. A threshold of 0.5 was applied to each ROI, and the data were dilated twice. For each individual ROI, an FA threshold of 0.15 was applied to ensure that we included only white matter in that region of interest despite the mask expansion.

6.3.4.3 Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy was used to non-invasively quantify metabolites in the whole brain. The MRS spin-echo chemical shift sequence was used with a voxel size of 20 mm x 23 mm x 13 mm and centered over the left and right dorsal hippocampi. The following sequence parameters were used in acquisition of spectroscopy data for the water-suppressed scan: TR = 1800 ms; TE = 68 ms; signal averages = 256, vector size= 1024. The following sequence parameters were used in acquisition of spectroscopy data for the non-water-suppressed scan: TR = 20000 ms; TE = 68 ms; signal averages = 1; vector size = 1024 point. Both water-suppressed and non-water-suppressed data were collected in institutional units, and all MRS data were analyzed with LC Model (version 6.3) using methods previously described (Radlowski et al., 2014). Limits were placed on MRS data for inclusion in the statistical analysis. Cramer-Rao lower bounds (i.e., % standard deviation) were calculated using LC Model and only metabolites with a standard deviation less than 20% were considered to have reliable quantitative results of absolute levels.

6.3.5 Hippocampal Gene Expression

Approximately 20 mg of hippocampal tissue were introduced in a Lysing Matrix D tube (MP Biomedicals, Santa Ana, California, USA), placed on ice, and 650µL of lysis buffer (Agencourt RNAdvance Tissue Kit, Beckman Coulter, Indianapolis, Indiana, USA) was added. Tubes were agitated for 2x1 minutes at speed 6

on FastPrep®-24 (MP Biomedicals, Santa Ana, California, USA), and 400µL of lysate were then extracted using the Agencourt RNAdvance Tissue Kit (Beckman Coulter, Indianapolis, Indiana, USA) following the manufacturer's recommendations. RNA were quantified using the Quant-iT™ RiboGreen™ RNA Assay Kit (Invitrogen, Carlsbad, California, USA) on a Spectramax M2 (Molecular Devices, Sunnyvale, California, USA). RNA quality assessment was completed using a Fragment Analyzer 96 with Standard Sensitivity RNA Analysis Kit (15 nt) (Advanced Analytical Technologies, Inc., Ankeny, Iowa, USA). Relative mRNA copy number on 93 genes was quantified using the NanoString nCounter™ system (NanoString Technologies Inc., Seattle, Washington, USA) according to the manufacturer's instructions using 100 ng of RNA as the starting amount. Relative copy number was obtained by normalizing the absolute copy number of each gene samples to the geometric mean of all housekeeping genes.

6.3.6 Statistical Analysis

Data analysis was conducted using the GLIMMIX procedure of SAS Enterprise Guide 7.1 (SAS Institute, Cary, NC, USA). All data were subjected to a two-way analysis of variance to assess the main effects of BMOS, HMO, and the interaction effect of BMOS and HMO. Two animals were removed from study due to failure to thrive (both provided BMOS + HMO). One piglet was removed from study at PND 2, however the second pig was removed after behavioral assessment and prior to MRI acquisition, and behavioral data was included in the analysis (all behavioral measures were within normal ranges). Cohort of pig was included in the model as a random variable. For all variables, observations with a studentized residual greater than |3| were considered outliers and removed from that variable only. For behavioral data, pigs that exhibited little exploration of either object (i.e., less than 2 seconds of exploration of the sample objects) were considered non-compliant and their recognition index was not measured in the test phase, but all other exploration measures were included for those subjects. Performance in the sample phases from the 1- and 48-h delay paradigms were averaged to create pooled sample phase exploration measures. To test for recognition memory, a one-sample t-test was conducted comparing the recognition index to a null mean of 0.5. Groups with a mean recognition index significantly above 0.5 as measured by a right-tailed one-sample t-test were considered to demonstrate recognition memory.

For individual brain region volume assessment, volume was expressed in both absolute (i.e., mm^3) and relative units (i.e., regional volume as a proportion of total brain volume, within subject). Gene expression data were standardized (mean of zero and standard deviation of one) and centered by the control group, thus all z-scores for the control group are zero. Statistical significance was defined at $p < 0.05$ (insignificant results not shown). Post-hoc comparisons for mean separation were conducted with a Tukey adjustment, and data are represented as least square means. Correlations between significant outcomes (MRI or gene expression) gene expression and the recognition index were conducted using the Pearson correlation coefficient for each diet and linear regression was used to assess the diet independent relationship between outcomes.

6.4 Results

6.4.1 Behavior

During the first habituation trial, piglets fed only HMO displayed less total movement in the arena as compared to controls ($p = 0.008$, Figure 6.1A). Piglets fed any diet containing BMOS (as BMOS or BMOS + HMO) displayed a lower increase in distance moved per minute across the duration of the first habituation trial than those not fed BMOS ($p = 0.027$, Figure 6.1B). There were no significant differences in any of the scored behaviors during the second habituation trial between groups (all $p > 0.223$). During the sample phase pigs fed diets containing HMO (as HMO or BMOS + HMO) spent more time investigating objects than those not fed diets containing HMO ($p = 0.032$, Figure 6.1C). The HMO group was able to show recognition memory after a 1-h delay (one-sample t-test, $p = 0.038$, Figure 6.1D), whereas only the BMOS + HMO group displayed recognition memory after a 48-h delay (one-sample t-test, $p = 0.045$, Figure 6.1D). Despite these differences, exploratory behavior (e.g., time spent investigating or number of visits to the sample or novel object) during the test phase was not significantly different between groups (all $p > 0.053$, data not shown).

6.4.2 MRI

A 3D surface rendering of the brain regions affected by the diet is shown in Figure 6.2. Interaction effects of BMOS and HMO were observed for absolute volumes of the caudate, hypothalamus, thalamus, and total grey matter, and a main effect of BMOS was observed for the lateral ventricle (all $p < 0.047$, Figure 6.3A-E). For all interaction effects observed for absolute volumes, pigs provided HMO demonstrated larger volumes of these regions if the diet also contained BMOS, whereas those consuming diets without HMO demonstrated smaller volumes if the diet also contained BMOS (Figure 6.3A, B, D, and E). As a proportion of total brain volume, interaction effects were observed for the caudate, lateral ventricle, and pons, with main effects of BMOS or HMO for the corpus callosum, lateral ventricle, left cortex, and right cortex (all $p < 0.042$, Figure 6.3F-K). There was no impact of diet for any DTI measure (AD, MD, RD, or FA, all $p > 0.063$, data not shown). Of the MRS outcomes, only glutathione, myo-inositol, N-acetylaspartate, and γ-amino butyric acid met criteria for inclusion, however none were altered by diet (all $p > 0.057$, data not shown).

6.4.3 Hippocampal Gene Expression

Numerous effects of diet were observed for measures of hippocampal mRNA expression. mRNA affected include: serotonin receptor 1 (*5HTR1*), the cholinergic receptor muscarinic 3 (*CHRM3*), the GABA type A receptor subunit β2 (*GABRB2*), the GABA type A receptor subunit ρ1 (*GABRR1*), the glycine receptor α4 (*GLRA4*), the glutamate ionotropic receptor NMDA type subunit 1 and subunit 2D (*GRIN1* and *GRIN2D*), the histone deacetylase 5 (*HDAC5*), the neural cell adhesion molecule 1 (*NCAM1*), the Nuclear Receptor

Subfamily 4 Group A Member 2 (*NR4A2*), the Solute Carrier Family 17 Member 6 (*SLC17A6*), the Solute Carrier Family 18 Member A2 (*SLC18A2*), the Solute Carrier Family 1 Member 7 (*SLC1A7*), the Solute Carrier Family 6 Member 3 (*SLC6A3*), and the synaptosome associated protein 25 (*SNAP25*) (all $p < 0.048$). Both BMOS and HMO groups showed downregulation of many genes compared to controls, whereas piglets fed BMOS + HMO displayed upregulation of those same genes. See Table 6.2 and Figure 6.4A for a description of the effects observed. After a Tukey adjustment for multiple comparisons, only *CHRM3*, *GABRB2*, *GLRA4*, and *SLC1A7* were significantly altered by diet (Table 6.2). To visualize the general trend for mRNA to be up- or downregulated as compared to controls, within diet, genes were ordered by descending Z-score and plotted on a heatmap (Figure 6.4B).

6.4.4 Correlation Analysis

To assess whether the outcomes were related or simply co-occurring, post-hoc correlations and regression were conducted against the recognition indices from the 1-h and 48-h delay trials against only those MRI and mRNA expression outcomes significantly affected by diet. A linear regression was conducted independent of diet (diet was not included in the model) to understand the overall trend between outcomes, and correlations were performed by diet group. Using the recognition index after a 1-h delay, four significant linear relationships were found between the recognition index and *GABRR1*, *GRIN1*, *HDAC5*, and *SLC1A7* (all $p < 0.028$, Figure 6.5). There were no relationships between significant MRI outcomes and the recognition index on either delay (all $p > 0.12$), and no relationships between significant gene expression outcomes and the recognition index after a 48-h delay (all $p > 0.11$).

6.5 Discussion

The objective of this study was to assess the effect of HMO, BMOS, and their combination on promoting recognition memory, and altering brain structure and hippocampal mRNA expression. While several studies have examined the effect of single oligosaccharide supplementation on cognition and brain development (Vázquez et al., 2015, Vazquez et al. (2016), Fleming et al. (2017), Schmidt et al. (2015), Savignac et al. (2016), Yen et al. (2017), Jia et al. (2016)), here we explored the individual and combined effects of BMOS and HMO.

The OS in human milk are primarily composed of neutral fucosylated OS, followed by neutral nonfucosylated, acidic (i.e., sialyllated), and acidic fucosylated OS (Kirmiz et al., 2018). Bovine milk is primarily composed of acidic and neutral nonfucosylated OS (Kirmiz et al., 2018). Although an oversimplification of their differences, bovine milk is largely absent in fucose containing OS and concentrated in sialic acid containing OS, whereas a significant portion of HMO contain fucose rather than sialic acid. Despite their differences in concentration, bovine milk does contain OS found in human milk (notably 3 or 6'SL, 2 or 3'FL, and LNnT (Aldredge et al., 2013)). Likewise, both human and bovine milk contain large quantities of fucose and sialic acid, respectively,

monosaccharides that have been linked to cognition. In the present study, the HMO provided were a combination of 2'FL and LNnT, whereas the BMOS were predominantly a mixture of galactooligosaccharides, with small amounts of sialyllactose. Therefore, pigs fed the HMO diet consumed a diet with OS content similar to that of human milk (OS were either neutral fucosylated or neutral nonfucosylated), and those fed BMOS consumed a diet similar to that of bovine milk (mostly neutral nonfucosylated with trace amounts of acidic OS).

Initial evidence demonstrated that impairment of fucosylation in the rat hippocampus attenuates memory retention during a discrimination task (Jork et al., 1986). Conversely, incorporation of fucose into glycoproteins in chicks increased after a passive avoidance task (Sukumar et al., 1980). These studies demonstrated that fucosylation plays a large role in memory processes. Since then, several studies have shown that administration of fucose or 2'FL impacts long-term potentiation (LTP) in the hippocampus, a molecular process that facilitates synaptic transmission and is believed to be the mechanism of long-term memory storage in the hippocampus (Baudry, 2001). Rats intrahippocampally injected with L-fucose or 2'FL demonstrated prolonged LTP duration compared to those injected with lactose (Krug et al., 1994). This effect was later replicated *ex vivo*, as bath application of L-fucose and 2'FL, but not D-Fucose or 3'FL, to rat hippocampal slices enhanced LTP by increasing the field excitatory postsynaptic potential and potentiation of the population spike amplitude (Matthies et al., 1996).

Although these studies demonstrated a strong link between fucose and memory function, the administration of fucose was independent of the diet, thus fucose and 2'fucosyllactose bypassed the gastrointestinal system. More recent work demonstrated that rats and mice fed 2'FL demonstrated increased and longer lasting potentiation of Schaffer collateral neurons in the CA1 region of the hippocampus (Vázquez et al., 2015). These animals also displayed increased performance on place learning, working memory, and fixed-ratio lever pressing tasks in an operant box, suggesting multiple cognitive domains were enhanced by 2'FL intake. A follow up study from the same lab demonstrated that rats fed L-fucose did not demonstrate the same enhancement of LTP as those fed 2'FL, and subdiaphragmatic bilateral vagotomy abolished the 2'FL dependent increase in LTP (Vazquez et al., 2016). This study provided two critical components to understanding the mechanism of action behind 2'FL mediated increases in cognition. First, ingestion of intact 2'FL and not L-fucose is required for cognitive enhancement. Second, the cognitive enhancing effect of 2'FL is mediated by the vagus nerve. Therefore, although 2'FL is absorbed by the gut as evidenced by increased concentrations in plasma and urine in infants (Marriage et al., 2015), it is likely that absorbed 2'FL does not act directly on the brain to promote cognition, but indirectly through vagal communication between the gut and brain. These data strongly suggest that although fucose is present as a glycoconjugate in the brain, the intake of fucose is not the primary driver of enhanced cognition.

If 2'FL acts via some prebiotic action to improve cognition, this would be consistent with the observation that monosaccharide-based OS with prebiotic activity (e.g., FOS or GOS) are also capable of improving

cognition (Kao et al., 2016). The hypothesis that all OS act via some specific prebiotic action to improve cognition provides a unifying explanation for the underlying mechanism but does not explain the differences observed between BMOS and HMO in the present study. If HMO are unique compared to other OS in the properties they impart to the immune system (Comstock et al., 2017), it follows that they might also be unique in their cognitive promoting abilities.

In the present study, piglets consuming only HMO were able to display recognition memory after a 1-h delay, whereas only those piglets consuming both HMO and BMOS were able to display recognition memory after a 48-h delay. Piglets consuming the control diet were unable to display recognition memory on either the 1- or 48-h delay. Although the effects on recognition memory are variable, piglets consuming any diet containing HMO displayed increased time investigating objects during the sample phase, clearly demonstrating that the presence of HMO in the diet increased object exploration. The effects on recognition memory are similar to those found previously when investigating the impact of oligofructose with or without 2'fucosyllactose on performance in the NOR task (Chapter 5). In both the present and former study, the ability to display recognition memory was dependent on both the time delay and OS content of the diet. Although data suggests that multiple OS may improve cognitive performance (Kao et al., 2016), it is becoming clear that not all OS have the same cognitive promoting capacity.

When we examined hippocampal mRNA expression, intake of BMOS or HMO produced significant downregulation of several genes, whereas the intake of both BMOS and HMO produced upregulation in those same genes (of those shown in Table 6.2, *GABRR1*, *GRIN2D*, *SLC18A2*, *SLC1A7*, and *SLC6A3* did not follow this trend). Genes affected were related to many processes, however after a post-hoc Tukey adjustment for multiple comparisons four genes were found most impacted by diet: *GABRB2*, *SLC1A7*, *GLRA4*, and *CHRM3*. As the genes affected represent several distinct processes and were not affected by the diet in any clear pattern, we conducted a diet-independent linear regression to assess whether they were related to recognition memory. Interestingly, no gene examined correlated with recognition memory after a 48-h delay, similar to a previous report from our lab (Chapter 5). Of all the genes affected shown in Table 6.2, four were significantly related to recognition index after a 1-h delay: *GRIN1*, *GABBR1*, *HDAC5*, and *SLC1A7* (Figure 6.5). The downregulation of these genes was related to increased recognition memory. When taking diet into account, the presence of a relationship was highly variable. The relationship between the recognition index and *GRIN1* was driven by the control group, the relationship between the recognition index and *HDAC5* was driven by the control and BMOS + HMO group, and only an overall relationship was observed between the recognition index and *GABRR1* or *SLC1A7* (Figure 6.5).

This is the second time we found that increased expression of *HDAC5* is related to a decreased recognition index (Chapter 5). With replication of that result, *HDAC5* may play a larger role in cognitive development than previously assumed. This may help explain why neither the BMOS nor BMOS + HMO group were able to display recognition memory after a 1-h delay, as pigs consuming any diet containing BMOS demonstrated

higher mRNA expression of *HDAC5* compared to controls. This may seem inconsistent with the observation that the BMOS + HMO group displayed recognition memory after a 48-h delay, however we found no relationships between mRNA expression and recognition memory after a 48-h delay. We had previously hypothesized that HDACs may be involved in cognition via the inhibitory action of short-chain fatty acids, fermentative end products of microbial digestion in the colon. Short-chain fatty acids are known to act indirectly via transcriptional regulation as HDAC inhibitors or directly to modulate satiety (Frost et al., 2014), neurogenesis (Kim et al., 2009), and blood-brain-barrier integrity (Braniste et al., 2014). However, despite the transport and absorption of butyrate and acetate in the blood and brain, we previously found little evidence that the concentration of short chain fatty acids in the colon, blood, or brain were related to recognition memory in pigs (Fleming et al., 2017). With the replicated finding that *HDAC5* is related to recognition memory, it is tempting put forth the hypothesis that *HDAC5* is a key regulator of cognitive development in context of OS consumption, however it is important to keep in mind that these two effects were simply co-occurring, and there may be an unmeasured variable that better explains the effect of oligosaccharide intake on cognition.

Similar to behavior and mRNA expression, consumption of HMO or BMOS did not produce consistent effects on the volume of various brain regions, rather the effects observed were specific to each brain region and varied between diets. On an absolute basis, a main effect of BMOS for the lateral ventricle and interaction effect of BMOS and HMO were observed for the grey matter, thalamus, caudate, and hypothalamus (Figure 3A-E). Relative to total brain volume, pigs fed any diet containing HMO demonstrated large volumes of the cortices and corpus callosum (Figure 6.3F-H). Additionally, a main effect of BMOS was found in the corpus callosum where BMOS-fed pigs demonstrated lower volume than pigs not fed BMOS (Figure 6.3H). There was an interaction of BMOS and HMO for the pons, lateral ventricle, and caudate. For the pons, supplementation with diets containing only BMOS or HMO increased the relative volume compared to controls and pigs fed BMOS + HMO, which demonstrated similar relative volumes (Figure 6.3I). In the lateral ventricle, both a main effect of BMOS and an interaction effect of BMOS + HMO were observed. Pigs fed diets containing HMO demonstrated the largest relative volume, with pigs fed BMOS + HMO the smallest (Figure 6.3J). Lastly, pigs fed BMOS + HMO demonstrated larger relative volumes of the caudate as compared to those fed only BMOS or HMO (Figure 6.3K).

Whether these changes are beneficial or detrimental is unclear, none of these changes appeared to be related to recognition memory when assessed via linear regression). Based on the absolute and relative volumes, the interaction effects show that the control and BMOS + HMO groups have similar values for almost all regions (with the exception of the lateral ventricle, cortices, and corpus callosum). Essentially, regional size was similar between pigs given no OS or both BMOS + HMO. An important confounder however is that the dosage of oligosaccharide was not controlled for. Therefore, pigs given HMO, BMOS, and BMOS + HMO consumed increasing levels of oligosaccharide, respectively. The pattern wherein pigs given HMO or BMO

differ from those provided the control diet or HMO + BMOS may be indicative of a dose effect, however, this pattern is not evident in either behavioral or mRNA expression data.

Past research from our lab has demonstrated that supplementation with sialyllactose had no effect on regional brain volumes but was able to alter the diffusivity of the corpus callosum and left hippocampus (Mudd et al., 2017b). When supplemented with a mixture of prebiotics, lactoferrin, and milk fat globule membrane, the grey and white matter concentrations were most notably altered in the left and right cortex (Mudd et al., 2016a). When consuming diets containing oligofructose, piglets demonstrated larger olfactory bulbs (Chapter 5). Herein we demonstrate an additional example where prebiotic supplementation has altered brain structure. Despite these changes, no differences were observed for spectroscopy or diffusion tensor imaging, suggesting that the diet did not affect white matter integrity or gross metabolic state.

Overall, the variability between studies in which brain regions and outcomes are altered by prebiotic consumption proves difficult to explain. Where the effects on recognition memory and even gene expression have shown effects replicated across studies, regional volumes of the brain affected by OS consumption is highly variable. As we captured a cross-sectional representation of neurodevelopment, further efforts to quantify the effect of OS consumption on neurodevelopment using a longitudinal study design may prove useful to identify the long-term effects of OS consumption.

6.6 Conclusion

To date, supplementation with oligosaccharides has generally been shown to either impart neutral or beneficial effects on cognition and brain development. Here, we investigated the impact of human and bovine milk oligosaccharides on cognition, structural brain development, and hippocampal mRNA expression. Although we show that supplementation with HMO and/or BMOS generated apparent improvements to behavioral performance, the various effects on regional brain volumes and hippocampal mRNA expression highlight the need to delineate the importance of such effects.

6.7 Tables

Table 6.1: Oligosaccharide content of the diets.*

Diet, g/L	Bovine Milk OS†	2'Fucosyllactose‡	Lacto-N-neotretraose‡	Lactose
Formulated				
CON	0	0	0	13.86
BMOS	12.4	0	0	1.47
HMO	0	0.98	0.48	12.4
BMOS + HMO	12.4	0.98	0.48	0
Analyzed				
CON	0	0	0	NQ
BMOS	5.79	0	0	NQ
HMO	0	0.81	0.42	NQ
BMOS + HMO	5.75	1	0.53	NQ

* Abbreviations: OS, Oligosaccharides; CON, control group; HMO, pigs fed human milk oligosaccharides; BMOS; pigs fed bovine milk oligosaccharides, BMOS + HMO, pigs fed both human and bovine milk oligosaccharides; NQ, not quantified.
† Nestlé Product & Technology center, Konolfingen, Switzerland.
‡ Glycom, Hørsholm, Denmark

Table 6.2: mRNA expression.[*†]

						p-value		
Measure	CON	BMOS	HMO	BMOS + HMO	Pooled SEM	BMOS	HMO	Int
$5HTR1^{\ddagger}$	0	-0.49	-0.69	0.06	0.31	0.654	0.808	0.039
$CHRM3$	0^{ab}	-0.59^{ab}	-0.70^{b}	0.39^{a}	0.29	0.366	0.612	0.004
$GABRB2$	0^{ab}	-0.31^{b}	-0.30^{b}	0.99^{a}	0.28	0.071	0.064	0.004
$GABRR1^{\ddagger}$	0	-0.12	-0.7	-0.61	0.31	0.959	0.047	0.724
$GLRA4$	0^{ab}	-0.45^{b}	-0.07^{ab}	0.70^{a}	0.32	0.548	0.048	0.027
$GRIN1^{\ddagger}$	0	0.45	0.02	0.53	0.37	0.035	0.818	0.888
$GRIN2D^{\ddagger}$	0	-0.62	-0.56	-0.05	0.34	0.822	0.979	0.037
$HDAC5^{\ddagger}$	0	0.62	0.17	0.87	0.35	0.015	0.428	0.876
$NCAM1^{\ddagger}$	0	-0.48	-0.5	0.21	0.33	0.682	0.743	0.041
$NR4A2^{\ddagger}$	0	-0.37	-0.52	0.56	0.30	0.211	0.480	0.015
$SLC17A6^{\ddagger}$	0	-0.58	-0.48	0.32	0.34	0.673	0.437	0.012
$SLC18A2^{\ddagger}$	0	-0.47	-0.99	-0.76	0.30	0.670	0.031	0.230
$SLC1A7$	0^{a}	-0.11^{a}	-0.14^{a}	-1.23^{b}	0.32	0.026	0.020	0.066
$SLC6A3^{\ddagger}$	0	0.28	-0.57	-0.62	0.34	0.689	0.018	0.593
$SNAP25^{\ddagger}$	0	-0.58	-0.54	0.32	0.30	0.615	0.536	0.015

[*] Abbreviations: CON, control group; HMO, pigs fed human milk oligosaccharides; BMOS; pigs fed bovine milk oligosaccharides, BMOS + HMO, pigs fed both human and bovine milk oligosaccharides; SEM, standard error of the mean; INT, interaction effect of HMO and BMOS; $5HTR1$, serotonin receptor 1; $CHRM3$, cholinergic receptor muscarinic 3; $GABRB2$, GABA type A receptor subunit $\beta 2$; $GABRR1$, GABA type a receptor subunit $\rho 1$; $GLRA4$, glycine receptor $\alpha 4$; $GRIN1$ and $GRIN2D$, glutamate ionotropic receptor NMDA type subunit 1 and subunit 2D; $HDAC5$, histone deacetylase 5; $NCAM1$, the neural cell adhesion molecule 1; $NR4A2$, nuclear receptor subfamily 4 group a member 2; $SLC17A6$, solute carrier family 17 member 6; $SLC18A2$, solute carrier family 18 member A2; $SLC1A7$, solute carrier family 1 member 7; $SLC6A3$, solute carrier family 6 member 3; $SNAP25$, synaptosome associated protein 25.

[†] Standardized values for mRNA expression (mean = 0, standard deviation = 1) centered by control group. Only measures significantly altered by diet are shown. Data were analyzed via two-way ANOVA with a post-hoc Tukey adjustment for multiple comparisons. Means without a common superscipt differ ($p < 0.05$)

[‡] Mean separation insignificant after Tukey adjustment.

6.8 Figures

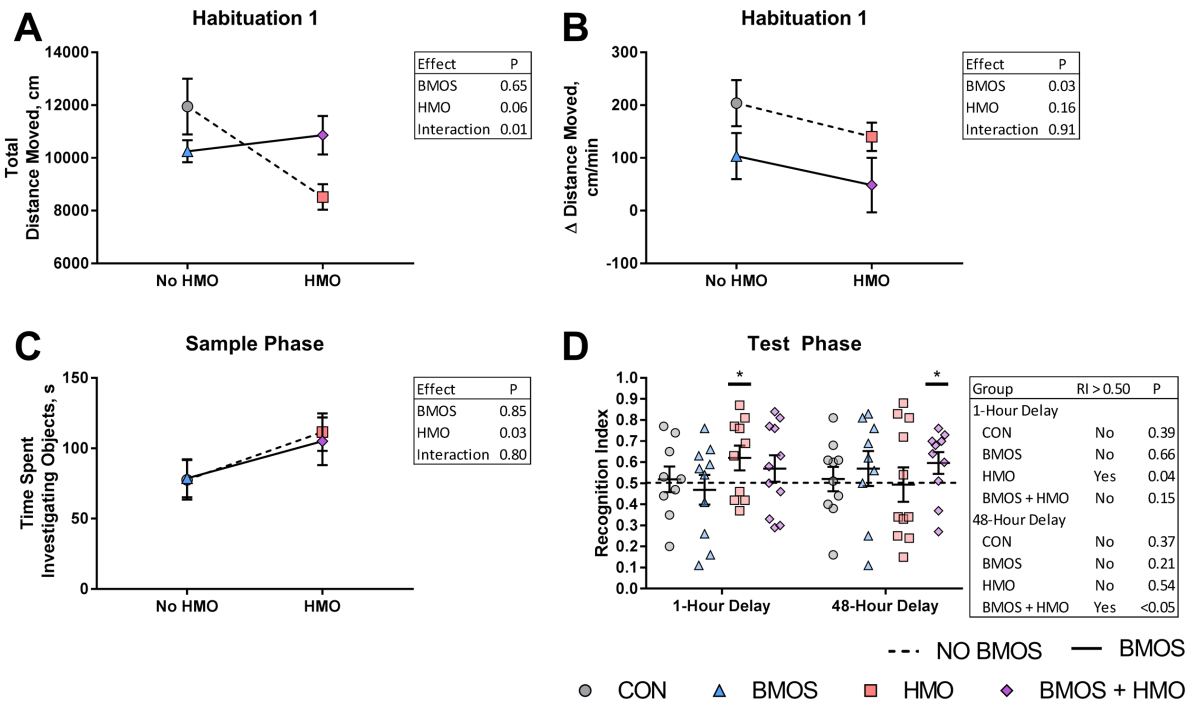

Figure 6.1: Performance during the novel object recognition task. **A)** An interaction effect was observed for total distance moved during the first habituation trial. **B)** During the first habituation trial, pigs fed diets containing BMOS showed a lesser increase in distance moved per minute than those not fed BMOS. **C)** During the sample phase piglets fed HMO in any diet spent more time investigating objects than those fed diets without HMO. **D)** Piglets fed the HMO-only diet were able to display a recognition index greater than 0.5 after a 1-h delay, however only those fed both BMOS and HMO were able to display a recognition index greater than 0.5 after a 48-h delay, as indicated by the asterisks.

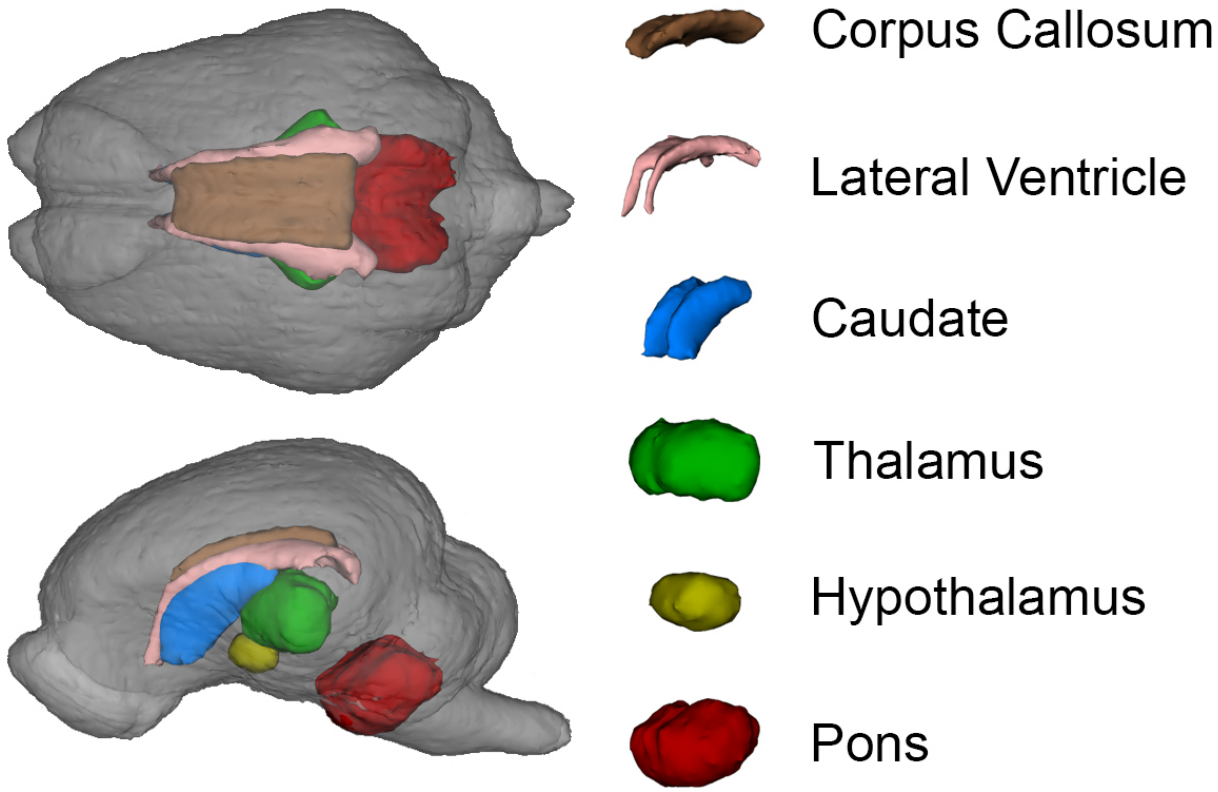

Figure 6.2: Representative 3D surface rendering of the brain from the pig brain atlas. Only brain regions altered by the diet are shown. Although affected by diet, the cortices and grey matter are not highlighted to allow visualization of subcortical structures.

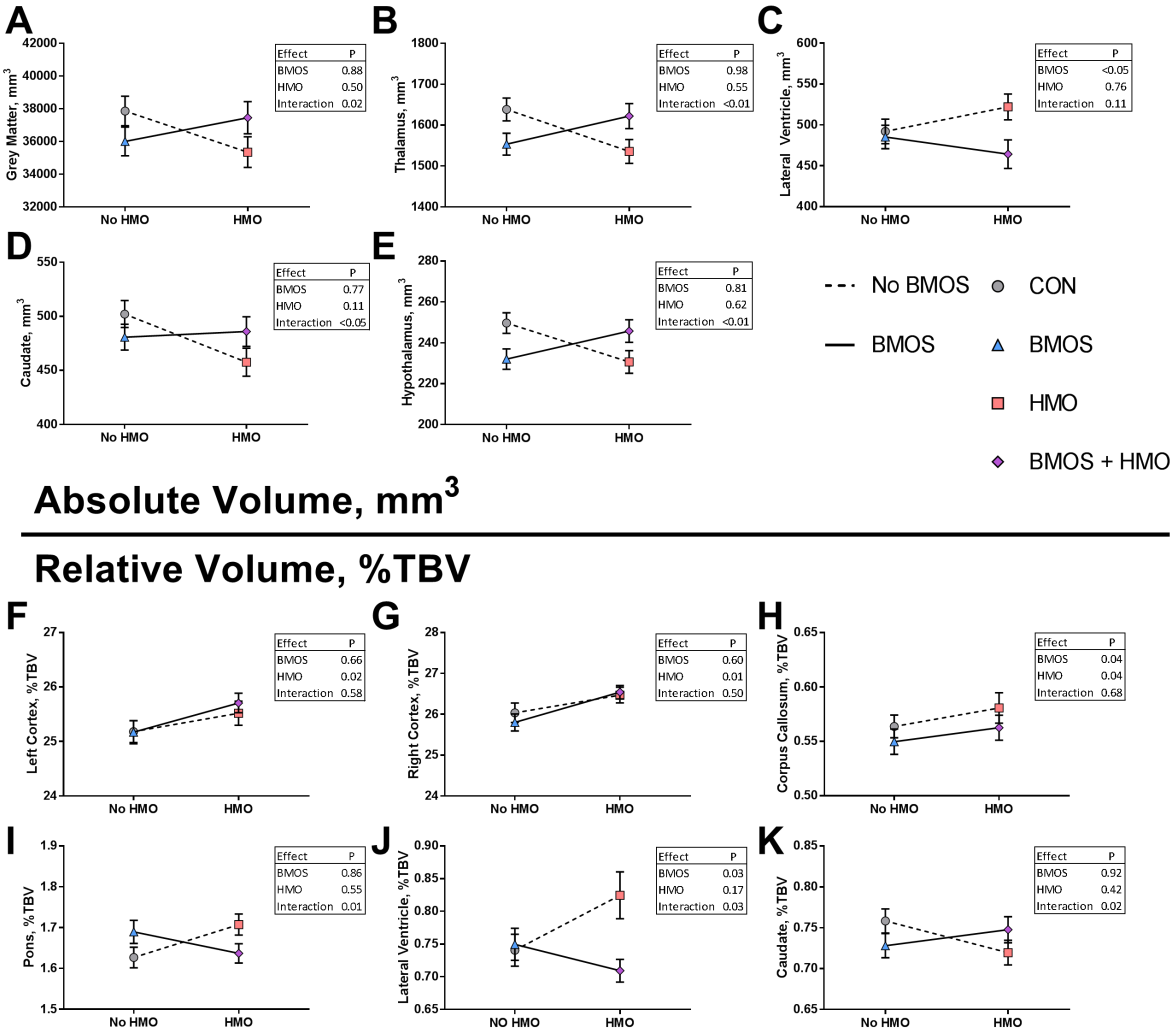

Figure 6.3: **A-E)** Absolute volume of regions affected by diet. In each case, (except **C**) an interaction effect was observed wherein pigs fed the CON or BMOS + HMO diet displayed similar absolute volumes compared to pigs fed either BMOS or HMO. **F-K)** Relative volumes of regions affected by diet. **F-G)** A main effect of HMO was seen for both the left and right cortices, pigs fed any diet containing HMO demonstrating larger relative cortices. **H)** Pigs fed diets containing HMO had a larger relative corpus callosum, whereas pigs fed diets containing BMO had a smaller relative corpus callosum. **I-K)** Similar to the effects seen for absolute volumes, interactions effects of diet for relative volumes of the caudate, lateral ventricle, and pons were observed, wherein pigs fed the CON or BMOS + HMO diet displayed similar relative volumes compared to pigs fed either BMOS or HMO.

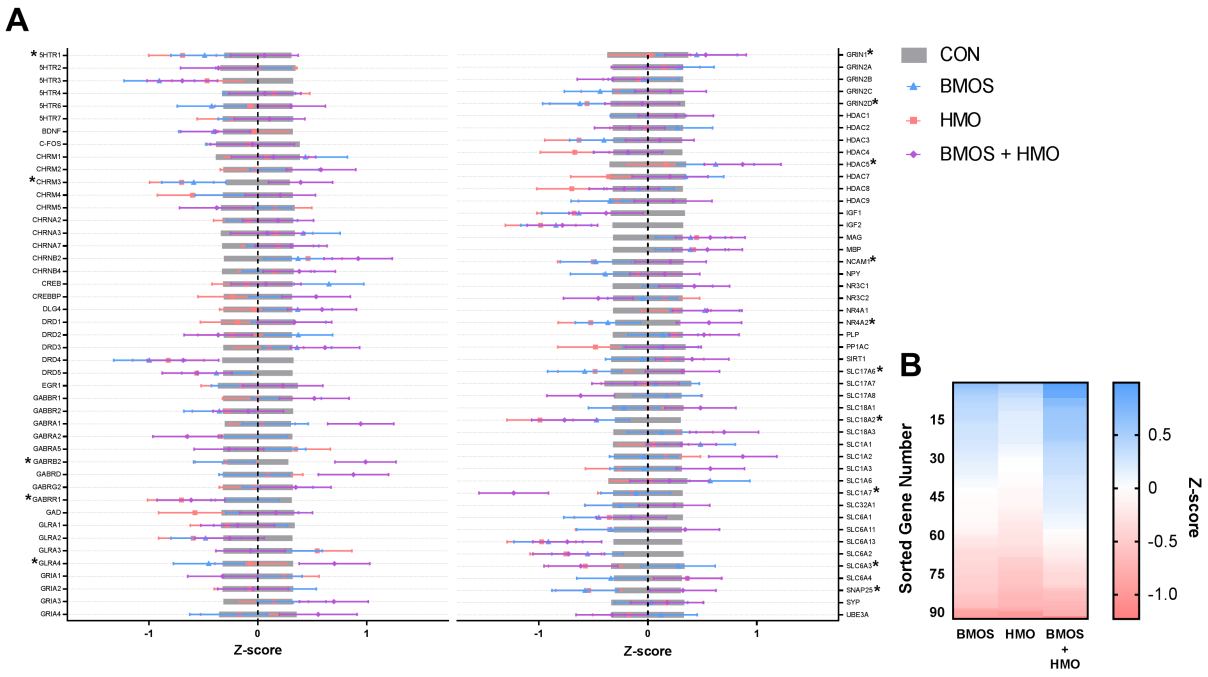

Figure 6.4: Hippocampal tissue was assessed for the mRNA expression of 93 genes. **A)** Figure depicts standardized data (mean = 0, standard deviation = 1) centered by control group. Values below zero indicate decreased expression compared to control, whereas values above zero indicate increased expression. Bars show mean + standard error, genes significantly impacted by diet are denoted by an asterisk. **B)** Genes were sorted in descending order by Z-score for each diet, visualizing the trend where the HMO group exhibited greater downregulation of mRNA compared to the BMOS or BMOS + HMO group. Abbreviations: CON, control group; BMOS, pigs fed a mixture bovine milk oligosaccharides; HMO, Pigs fed 2'fucosyllactose + Lacto-N-neotetraose; BMOS + HMO, pigs fed both human and bovine milk oligosaccharides.

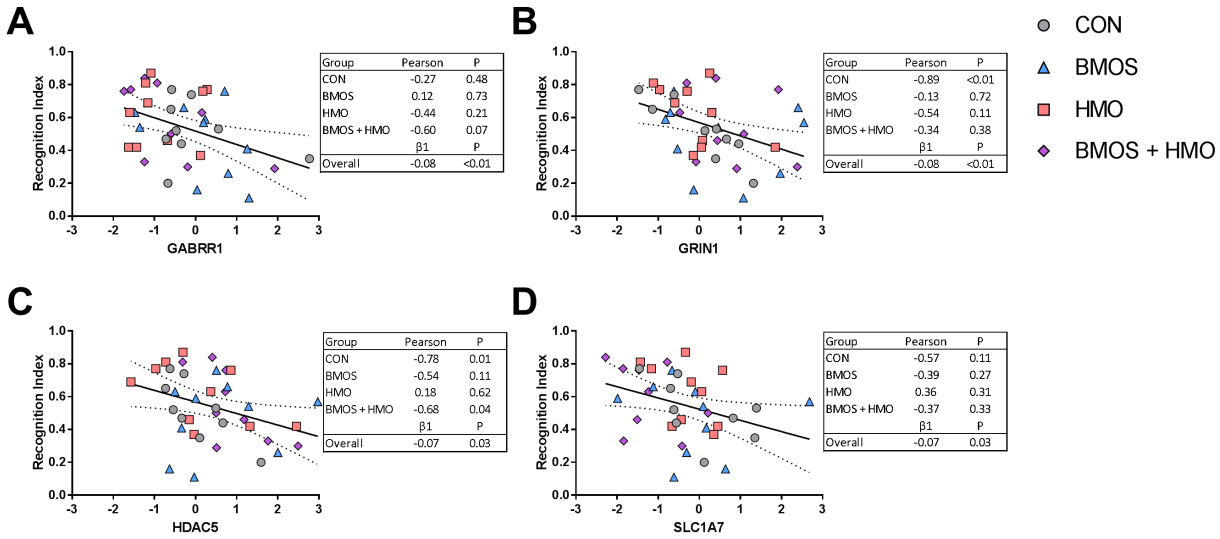

Figure 6.5: Of the variables affected by diet, four were shown to correlate with the recognition index after a 1-h delay. No relationships were found between MRI or gene expression outcomes affected by diet and the recognition index after a 48-h delay. The relationships observed were not present equally across all diets, however study-wide all were negatively related to the recognition index.

Chapter 7

Examining the Relationship between Volatile Fatty Acids and Recognition Memory

7.1 Abstract

Consumption of prebiotics and probiotics have been found to have substantial effects on host physiology, extending even to the behavior and affective states of the host. The mechanisms behind such effects are largely unknown, and experimental work defining the nature of the gut-brain-axis is in its infancy. The gut has several known roles that may link it to the brain's function. Fermentation and production of microbial metabolites, vagal innervation, and immune protection have been proposed as likely mediators within the gut-brain-axis. Here, a retrospective analysis was done to examine the relationship between colonic volatile fatty acids (VFA) and recognition memory. Data were collected from multiple studies assessing the impact of oligosaccharides on behavioral performance on the novel object recognition task and colonic VFA. Correlation and regression analyses were performed to assess the presence of relationships between recognition memory and VFA, the impact of colonic region on this relationship, and whether or not observed relationships were mediated by catecholamines in the brain. Relationships between memory and VFA were only found in the ascending colon, and not the cecum or feces ($p < 0.05$). Relationships were repeatedly observed among the branched chain fatty acids isovalerate and isobutyrate, with each BCFA negatively correlated with recognition memory ($p < 0.05$). Lastly, catecholamines were found to have little correlation with recognition memory ($p > 0.05$) and did not mediate the relationship between VFA and memory. These findings point towards diet and region-specific relationships within the gut-brain-axis. The sensitivity of these relationships to diet

highlight the difficulty of proposing a unifying theory to explain communication within the gut-brain-axis and point towards the need for better understanding of gut-brain physiology.

7.2 Introduction

Though it is becoming increasingly clear that prebiotics and probiotics can induce improvements in cognition and affective states (Kao et al., 2016, Sampson and Mazmanian (2015)) the mechanisms behind such actions are unknown. As discussed in Chapter 3, short chain fatty acids may act on the brain by modulating histone acetylation (Stilling et al., 2016) and blood brain barrier permeability (Braniste et al., 2014). MacFabe et al., (Macfabe, 2012) postulate that SCFA, specifically propionate, may also play a role in autism-like behaviors. However there has been little experimental work examining the role of endogenous VFA in altering behavior.

Hanstock et al., (Hanstock et al., 2004) fed 36 male Wistar rats commercial chow containing soluble carbohydrates (wheat), fermentable carbohydrates (cooked rice), or a control chow. Both test diets contained less crude fiber, fat, and protein than the control group, however digestible energy was similar between diets. Rats fed soluble or fermentable fiber demonstrated increased cecal VFA 3- and 21-hr post-prandially. After testing on a social interaction and light/dark box task (a test of anxiety), it was revealed that cecal lactic acid was positively associated with anxiety and aggression parameters. After including prefrontal cortex dopamine concentrations as an independent variable, cecal propionate was also shown to positively relate to fighting frequency. The authors suggest that although fiber intake is associated with increased butyrate and may reduce the risk for colon cancer, the effect of other VFA on behavior is unknown and no causal links exist between fermentation and behavior. A follow-up study (Hanstock et al., 2010) employing the same study design found that plasma lactate, but not cecal lactate, was negatively related to recognition memory. Mice with higher levels of plasma lactate demonstrated worse performance on the novel object recognition task, however there was no difference in recognition memory between dietary groups. Given significant differences in protein and fat content of the diet, it is difficult to state that such relationships are related to fiber intake (which was actually lower in both test groups compared to controls). Furthermore, regression was performed without diet included in the model, so it is unclear if such relationships are present equally between diets. Taken together, these two studies provide preliminary evidence that there is a relationship between lactate and behavior, and this relationship is dependent on sample region collected (i.e., cecum, feces, or blood). Overall, there were few or no relationships with acetate, butyrate, and propionate with behavior.

In an invasive study, twice daily intracerebroventricular infusions of propionic acid for 7 days reduced time spent with novel rats and impaired performance on the T-maze task during the reversal phase, indicating less socialization and impaired cognitive flexibility (Macfabe, 2012). Propionic acid administration also increased lipid and protein oxidation and neuroinflammation. The effect on socialization, cognition, and metabolism are proposed by the authors as related to Autism Spectrum Disorder, where behavioral conditions may arise from improper metabolism of propionic acid. The authors suggest these results may be related to organic

acids (primarily succinate) influence on neurotransmitter synthesis, calcium influx, pH maintenance, lipid metabolism, mitochondrial function, gap-junction-dependent intercellular gating, immune activation, and gene expression (Macfabe, 2012).

Given the relatively sparse amount of data regarding the relationship between VFA and behavior, a retrospective analysis was conducted on two previous studies (Fleming et al., 2017, Fleming et al. (2018)) and those in Chapters 5 and 6. The goal of this analysis was to answer four questions: Does bodyweight and oligosacchride intake predict recognition memory, are VFA related to recognition memory, is this relationship dependent on colonic region, and do catecholamines mediate the relationship between volatile fatty acids and recognition memory?

7.3 Methods

Methods describing the experimental design, animal housing, and sample collection have been previously reported in published articles (Fleming et al., 2017, Fleming et al. (2018)) and in Chapters 5 and 6, and will be explained in brief here.

7.3.1 Animals and Housing

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. Beginning at postnatal day 2, 24 naturally farrowed intact male pigs were sourced from a commercial herd and artificially reared to postnatal weeks 3 or 4. Pigs were allotted to dietary groups and counterbalanced for equal representation of litter and average body weight across groups. Prior to placement in the artificial rearing system, pigs were administered 5.0 mL of *Clostridium perfingens* antitoxin C + D per the manufacturer's recommendations (Colorado Serum Company, Denver, CO, USA). All pigs were housed in master caging units that contained six individual stainless-steel cages (L x W x H of 87.6 x 88.9 x 50.8 cm) with clear, polycarbonate facades on three sides of the cage and vinyl-coated, expanded-metal flooring (Tenderfoot^{TM}, Minneapolis, MN). The master unit was designed such that there were three separate levels each with two individual pig cages on each level. Pigs on each level shared a common wall containing holes to permit pigs to see, smell, hear, and minimally touch one-another. A towel and toy were included in each cage to provide enrichment, all pigs were removed from cages and allowed to socialize with each other for approximately 30 min each day, and all pigs were allowed *ad libitum* access to water at all times. Ambient room temperature was maintained between 27°C-29°C and a 12 h light/dark cycle was maintained with light from 0800 to 2000h.

7.3.2 Behavior Analysis and Sample Collection

Pigs were tested on the novel object recognition task using a 2-day delay to assess hippocampal-dependent recognition memory. Testing consisted of a habituation phase, a sample phase, and a test phase. During the habituation phase, each pig was placed in an empty testing arena for 10 min each day for two days leading up to the sample phase. In the sample phase, the pig was placed in the arena containing two identical objects and given 5 min for exploration. After a delay of 45-48 hours, the pig was returned to the arena for the test phase. During the test phase, the pig was placed in the arena containing one object from the sample phase as well as a novel object and allowed to explore for 5 min. Between trials, objects were removed, immersed in hot water with detergent, and rubbed with a towel to mitigate odor, and the arena was sprayed with water to remove urine and feces. Objects chosen had a range of characteristics (i.e. color, texture, shape, and size), however the novel and sample objects only differed in shape and size. Only objects previously shown to elicit a null preference were used for testing (Fleming and Dilger, 2017). Task order was counterbalanced between replicates. Habituation trials began at: PND 17 (Fleming et al., 2018), 22 (Chapters 5 and 6), or 25 (Fleming et al., 2017). The recognition index, the proportion of time spent with the novel object/location compared to total exploration of both objects, was used to measure recognition memory. A recognition index significantly above 0.50 demonstrates a novelty preference and thus recognition memory.

At end of study pigs were anesthetized by intra-muscular injection at 0.03 mL/kg bodyweight using a telazol:ketamine:xylazine solution [50.0 mg tiletamine plus 50.0mg zolazepam reconstituted with 2.50 mL ketatamine (100 g/L) and 2.50 mL xylazine (100 g/L); Fort Dodge Animal Health]. Upon verifying anesthetic induction, piglets were euthanized via intracardiac administration of sodium pentobarbital (86.0 mg/kg bodyweight; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA. Samples collected and stored for subsequent analyses included brain tissue (hippocampus, striatum, and medial prefrontal cortex), feces, and luminal contents from the large intestine. All samples except luminal contents from the large intestine were snap frozen in liquid nitrogen and kept at 80°C for long-term storage. Samples from the large intestine included the cecum (anterior to the ileocecal junction), ascending colon (within approximately 15.24cm posterior to the ileocecal junction), and rectum (these were considered fecal samples). Cecal and ascending colon contents were weighed and immediately preserved for quantification of volatile fatty acids using acidification with an equal volume of 2 N HCl (fecal samples were thawed later and acidified with 6.25% m-Phosphoric acid). Samples were collected in duplicate to quantify both dry matter content (without acidification) and volatile fatty acid concentration. Frozen brain tissue was mechanically homogenized in liquid nitrogen using a Freezer/Mill (SPEX, USA) and the powdered samples stored at 80°C.

7.3.2.1 Volatile Fatty Acid Analysis

After acidification, samples were sonicated to homogenize contents and allowed to precipitate at room temperature for 30 minutes and centrifuged for ten minutes at 15,500 g. Supernatant was extracted and

reconstituted with equal volume of ethyl acetate and vortexed for two minutes. Samples were then centrifuged again for ten minutes at 16,500 *g* and the supernatant was used for analysis via gas chromatography. Briefly, concentrations of acetate, propionate, and butyrate were determined in the supernatant of the tubes using a Hewlett-Packard 5890A Series II gas chromato- graph (Palo Alto, CA) and a glass column (180 cm x 4 mm i.d.) packed with 10% SP-1200/1% H3PO4 on 80/100 mesh Chromosorb WAW (Supelco Inc., Bellefonte, PA). Nitrogen was the carrier gas with a flow rate of 75 mL/min. Oven temperature, detector temperature, and injector temperature were 125, 175, and 180°C, respectively. Short-chain fatty acid concentrations were corrected for the quantities of SCFA produced in the blank tubes.

7.3.2.2 Catecholamine Analysis

Absolute quantification of the following catecholamines were conducted in duplicate using validated liquid chromatography methods: dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine, epinephrine, 5-hydroxytryptamine (serotonin) and 5-hydroxyindoleacetic acid (HIAA). In brief, high-performance liquid chromatography (HPLC) methods based on the Dionex Application Note 228 were used for rapid and sensitive separation and quantification using electrochemical detection (Dionex, 2009). Method B of this procedure was employed to simultaneously quantify all the aforementioned biochemicals in brain samples, and extraction procedures were optimized for extraction of catecholamines from pig brain tissue samples.

7.3.3 Dietary Treatments

Diets provided are described in Table 7.1. For all studies, milk replacer was reconstituted fresh daily by mixing 200g of powdered milk replacer with 1 liter of potable tap water. Milk was provided at restricted feeding times (Fleming et al., 2017) or *ad libitum*. If provided at restricted times, milk was provided at a rate 285 and 325 mL of reconstituted milk replacer per kg BW beginning on PND 2 and 8, respectively, across 10 feeds approximately every 2 hours from 1000 h on one day to 0400-600 h the subsequent day ((Fleming et al., 2017) and Chapters 5 and 6). All diets were formulated to meet dietary requirements (National Research Council, 2012) and nutrient compositions are shown in Tables 7.2 and 7.3. Growth, milk intake, and oligosaccharide intake can be found for each diet in Figure 7.1.

7.3.4 Statistical Analysis

The dependent variable for all analyses was the recognition index (RI) from the novel object recognition task. The recognition index is the proportion of time spent visiting the novel object as compared to both the sample and novel object. Values above 0.50 are indicative of the subject having a novelty preference, whereas those below 0.50 are indicative of a familiarity preference. Thus, recognition memory is inferred when a subject-displays either preference. In previous sections, all improvements in novel object recognition

task were related to increases in the RI (shown in 7.4), thus the assumption will be made that increases to the RI are associated with improved recognition memory. The independent variables included body weight, milk intake, acetate, propionate, butyrate, isovalerate, valerate, isobutyrate, and percent dry matter. All VFA were expressed relative to the dry matter content of the sample. As an exploratory tool, Pearson correlations were constructed and mapped onto networks demonstrating positive/negative correlations (indicated as blue and red), and the significance of each relationship is indicated by the thickness of each line. All independent variables were grouped by diet and regressed against the recognition index. Due to abnormally low dry matter content, 2 of 6 cohorts were dropped from the analysis for diets in the "milk oligosaccharide" group (CON.MO, BMOS, HMO BMOS + HMO, OF, and OF + 2'FL groups). These groups only contained 6-8 pairwise observations for each independent and dependent variable, and thus low power for detecting significant correlations. Mediation analyses were performed using the R package `mediation` to assess if specific catecholamines mediated effects between volatile fatty acids and the recognition index. Nonparametric bootstrapping with a simulation size of 2000 was used for variance estimation using the percentile method.

7.4 Results

7.4.1 Aim 1: Do bodyweight or milk intake predict recognition memory?

Mean study body weight and mean daily milk intake were regressed against the recognition index in all groups. Body weight was not related to the recognition index in any group, however the OF group demonstrated trending significant for a weak correlation (b_1 = -0.127, SE = 0.06, $p = 0.06$) (Table 7.4 and Figure 7.2. Given milk intake is a function of body weight for those groups that were restricted fed (all except CON.SL and PDX-GOS + SL), there was no difference between body weight and milk intake regression output (Figure 7.3. For the CON.SL and PDX-GOS + SL groups that were fed *ad libitum*, there was no relationship between milk intake and the recognition index (all $p > 0.43$).

7.4.2 Aim 2: When are volatile fatty acids involved in recognition memory?

As shown in Figures 7.6 and 7.7, several relationships exist but these are highly dependent on the diet. From (Fleming et al., 2017), butyrate, isovalerate, and isobutyrate are related to the RI in pigs fed PDX-GOS, but no VFA are related to the RI in the control group. In a follow-up study investigating sialyllactose (Fleming et al., 2018), the control group (CON.SL) received the same diet as the PDX-GOS group from (Fleming et al., 2017). This replicated the effect shown with isobutyrate and isovalerate, but not butyrate. From Chapters 5 and 6, few correlations were present between VFA and RI in pigs fed various milk oligosaccharides. Isovalerate was again shown negatively related to RI in the group fed BMOS + HMO, but no other correlations existed except for DM, acetate, and propionate in the group fed OF.

7.4.3 Aim 3: Does colonic region affect the relationship between VFA and recognition memory?

Given the low sample size, diets from chapters 5 and 6 were omitted from this analysis. Here, we investigated whether the relationship was sensitive to cecal, ascending colon, or fecal samples (data from Chapter 4 (Fleming et al., 2018) only contained ascending colon and fecal samples). As shown in Figure 7.8, the concentration of VFA decreased from proximal to distal colon as the dry matter content increased. There were no relationships between any VFA and the RI in the cecum or feces, however fecal dry matter was positively related to RI. All existing relationships only existed in the ascending colon (Figures 7.8) and each of these relationships were specific to those fed PDX-GOS. From Chapter 4 (Fleming et al., 2018), this effect is replicated, with isovalerate and isobutyrate in the ascending colon but not feces related to the RI (Figure 7.9). Given high collinearity between isovalerate and isobutyrate, they show nearly the same relationship with the RI. Interestingly, these relationships were found in pigs fed PDX-GOS but disappeared in those fed PDX-GOS + SL. Statistics for each region can be found in Tables 7.5-7.7.

7.4.4 Aim 4: Do catecholamines mediate the relationship between VFA and recognition memory?

Data from Chapter 3 (Fleming et al., 2017) contained concentrations of catecholamines in the striatum, medial prefrontal cortex, and hippocampus. To investigate if catecholamines mediate the effects of VFA on recognition index a correlation network and mediation analyses were constructed. As shown in the correlation networks, few catecholamines were related to the RI in the control group (Figure 7.10) and no catecholamines were related to the RI in the group fed PDX-GOS (Figure 7.11 and Tables 7.8-7.9). Both isovalerate and isobutyrate were positively related to striatal serotonin and its metabolite 5-HIAA, whereas butyrate was related to striatal epinephrine. Given these catecholamines were not related to the RI, mediation analyses confirmed that the relationship between isovalerate and memory was not mediated by serotonin (Table 7.10), nor was the relationship between butyrate and memory mediated by epinephrine (Table 7.11).

7.5 Discussion

Here we found VFA, particularly BCFA, were predictive of recognition memory. The VFA in the ascending colon were far more predictive of recognition memory than those in the cecum or feces. Importantly, these relationships were highly dependent on the diet and region of the colon. This in part replicates the findings by Hanstock (Hanstock et al., 2004, Hanstock et al. (2010)) demonstrating that lactate is inversely related to anxiety, aggression, and recognition memory. While Hanstock did not measure BCFA, they also found few relationships with acetate, propionate, or butyrate with behavior. More importantly, between both the present analysis and Hanstock, VFA were always shown to be inversely related with positive behaviors.

In attempt to assess the possible existence of a dose-response effect of oligosaccharide intake, we regressed body weight and milk intake against the recognition index. Given most studies we investigated employed a restricted feeding paradigm, oligosaccharide and milk intake were direct functions of body weight. However the CON.SL and PDX-GOS + SL group were fed *ad libitum*, thus within-group variability existed in oligosaccharide intake. Regardless, we found little evidence that either body weight or oligosaccharide intake are related to recognition memory. At best, the OF + 2'FL group, a restricted fed group, was shown to weakly negatively predict the recognition index 7.5. Beyond our work, there has been relatively little investigation into the dose-response effect of prebiotics, with no evidence stating such effects with regard to cognitive outcomes. In a review, do Carmo et al., (do Carmo et al., 2016) detail the existing literature for polydextrose in relation to gastrointestinal outcomes. Although different doses have been used between studies, there have not been studies examining more than two doses at a time. While different doses have been used across different studies, many of these studies used different animal models, experimental designs, and outcomes.

Investigations into the relationship between fermentation and behavior have been explored since the late 1970's (Willard et al., 1977), despite little progress being made since then. Cecal-fistulated horses were fed hay, a corn/oat based-feed, or corn/oat based-feed in addition to cecal infusions of sodium carbonate. Those fed the corn/oat-based diet spent more time chewing wood and in coprophagy than those fed hay, and cecal infusions of sodium carbonate reduced these behaviors and increased cecal pH closer to those fed hay. Cecal propionate and lactate were found positively associated with time spent eating, whereas cecal acetate was inversely related with time spent eating. The authors suggest that increased cecal acidity may influence behavior towards wood chewing and coprophagy (Willard et al., 1977). These data provided early evidence that a link between fermentation and behavior may exist. Lactate, which is inversely related to SCFA production (Macfarlane and Macfarlane, 2003), intravenously infused into patients susceptible to panic attacks can induce panic attack episodes, suggesting a role for circulating lactate in altering anxiety related behavior (Nutt and Lawson, 1992). However, such experiments were not focused on the role of endogenously and microbially produced lactate in the gut. Regardless, such findings are replicated by Hanstock who found that cecal lactate is related to anxiety, aggression, and increased plasma lactate impairs recognition memory (Hanstock et al., 2004, Hanstock et al. (2010)). Taken with evidence demonstrating intracerebroventricular infusions of propionate induce autism-like behaviors (Macfabe, 2012), these data suggest that fermentation products are detrimental to cognition.

The negative effects of VFA shown in these studies is counter to some of the mechanisms believed to improve cognition and affective states when prebiotics and probiotics are consumed (Kao et al., 2016, Sampson and Mazmanian (2015)). If increased fermentation products are related to increased anxiety, aggression, and impaired memory, it seems unlikely that production of VFA after the consumption of prebiotics is likely to improve cognition. However, a reasonable shortcoming of these studies is the inability to track the flux of

VFA throughout an organism. Reduced concentrations are likely reflective of a combination of alterations to production, absorption, and cross-metabolism by other bacteria. A human study found that fecal acetate is inversely related to acetate absorption in the rectum and distal colon (Vogt and Wolever, 2003). Fecal acetate was measured from voided samples and human participants were then infused with acetate rectally. Rate of disappearance, and thus absorption, of acetate was strongly related to fecal acetate (r = -0.834). The authors suggest that fecal acetate is more likely a measure of the absorptive capacity of an individual rather than the level of production. Therefore, if increased colonic VFA are related to increased anxiety, it may be an error of absorption or metabolism rather than increased production, however such a theory is conjecture.

The finding that the relationships found were specific to the ascending colon, and not the cecum or feces, was unexpected. The cecum is the site of greatest fermentation, where the highest concentrations of VFA can be found (Figure 7.8), whereas feces contain the fewest VFA. The observation that each region of the colon harbors variable concentrations of VFA is unsurprising. It has long been known that different compartments of the colon (i.e., cecum, proximal, and distal colon) contain different concentrations and molar ratios of short-chain fatty acids (Edwards and Eastwood, 1995), which has significant effects on the prediction of *in vivo* concentrations (Millet et al., 2010). Beyond VFA, metabolomic analyses have found that while both the cecum and feces contain similar metabolites, the difference in concentration in each region is largely different (Zeng et al., 2015). Although a simplification, the cecum tends to contain carbohydrate and amino acid metabolites whereas feces contain greater fatty acid metabolites (Zeng et al., 2015). Such changes may be a causally or coincidentally related to the differences in microbial diversity in the cecum and feces. Phylogenetic diversity is less variable in fecal samples and have been shown to be less phylogenetically diverse and contain less *Tenericutes* and *Spirochaetesi* than cecal samples (Panasevich et al., 2018). Beyond microbial and metabolic differences, the vagus nerve directly connects the enteric and central nervous system, and known differences in innervation along the colon exist.

Retrogradely labelled neurons in the dorsal motor nucleus showed that the celiac and accessory celiac branches of the vagus nerve innervate the cecum highest, followed by the ascending colon and to a lesser extent the transverse and descending colon (Altschuler et al., 1993). This was confirmed by a later assessment demonstrating vagal intraganglionic laminar endings (IGLEs, one of two vagal afferent processes, the other being intramuscular arrays (IMAs)) innervate the colon greatest in the proximal colon, with the least innervation in the distal colon (Wang and Powley, 2000). However, there is a local maximum at the start of the mid-colon (approximately 55mm distal to the rat cecum), greater than that of the proximal or distal colon (Wang and Powley, 2000). IGLEs are postulated to have both chemo-sensory (Neuhuber, 1987) or mechano-sensitive roles in the colon (Kressel and Radespiel-Tröger, 1999). While VFA can direct vagal afferent neurons directly (Forsythe et al., 2014), the function of sensation of VFA in the colon is not known. Regardless of the true function of the vagus nerve, it has been demonstrated that vagotomized rats do not

derive the same benefits towards learning and memory or anxiety that non-vagotomized rats do (Vazquez et al., 2016, Lyte et al. (1998), Bravo et al. (2011)). If prebiotics and probiotics were to exert their beneficial actions after absorption and distribution of microbial metabolites, it seems unlikely that vagotomy should have such a strong effect. Together with the site-specific correlations found in the current analysis, the data suggest microbial metabolites play a role in a local, rather than systemic, manner.

We investigated whether catecholamines play an important role in mediating the effect of VFA on memory, however found little evidence of such an effect. In pigs fed PDX-GOS, no catecholamines measured were related with memory, however in the control group the RI was positively related with striatal dopamine and DOPAC. Mediation analyses were focused on the recognition index as a dependent variable, however as shown by Figures 7.10 and 7.11, there were many relationships between both VFA and catecholamines and indices of exploratory behavior in both the control and PDX-GOS fed groups. Despite the recognition index being calculated from time spent investigating both sample and novel objects, there did not appear to be strong relationships between exploratory behavior and recognition memory, suggesting the two variables are distinct. Thus, while catecholamines and VFA affected exploratory behavior, they had minimal effects on memory itself (with the exception of those discussed in the present results).

7.6 Limitations

Unfortunately, sample collection procedures for volatile fatty acid analysis were not standardized across all studies and samples (cecal and ascending colon contents were acidified pre-thaw whereas fecal samples acidified post-that). Furthermore, two different base formulas were used across some of the diets. Together with the use of multiple oligosaccharides included in the diets at different concentrations confounds the ability to make strong generalizations. The high specificity of the diet with respect to which effects were observed supports this limitation. Furthermore, the present studies were not designed to assess dose-response effects, and while multiple doses were used a dose-response effect could not be established. However, supporting the validity of these findings is the use of the same behavioral procedures used across all studies. Given high variability in both behavioral outcomes and volatile fatty acid concentrations, future studies should seek to robustly control for the dietary ingredients used, form of dietary test articles, and concentrations used both within and across studies. Lastly, while most of the significant correlations were moderate-to-strong ($r > 0.50$), greater sample size would allow the detection of weaker relationships.

7.7 Conclusions

Regression analysis found that few VFA are related to recognition memory. The branched chain fatty acids isovalerate and isobutyrate were consistently found negatively related to recognition memory, and this effect was specific to those VFA in the ascending colon. Catecholamines were not found to be related to recognition

memory, and thus did not mediate the effects of VFA on recognition memory. These data point towards the need for a better understanding of the local action of VFA in the colon, specifically related to vagal innervation of the colon and sensation of microbial metabolites.

7.8 Tables

Table 7.1: Diet groups

Diet*	Article	Dose, g OS/L milk†	Total OS, g/L	Feeding Paradigm	MR Dose per day	Feeding Period
CON.PDX-GOS	Control containing trace levels of Sialyllactose	0 added OS and 0.058 Sialyllactose	0.058	Restricted	285-325 ml MR/kg BW	PND 2-33
PDX-GOS	Polydextrose and Galactooligosaccharide	1.8 PDX and 2.1 PDX GOS each	3.958	Restricted	285-325 ml MR/kg BW	PND 2-33
CON.SL	Polydextrose and Galactooligosaccharide	1.8 PDX and 2.1 PDX GOS each	3.958	*Adlibitum*	Variable	PND 2-22
PDX-GOS + SL	PDX-GOS + Sialyllactose	PDX-GOS + 0.38 m Sialyllactose	4.274	*Adlibitum*	Variable	PND 2-22
CON.MO	Milk oligosaccharide control	0 added OS and 0.058 Sialyllactose	0.000	Restricted	285-325 ml MR/kg BW	PND 2-33
OF	Oligofructose	3.62 OF	3.620	Restricted	285-325 ml MR/kg BW	PND 2-33
OF + 2'FL	OF + 2'Fucosyllactose	3.41 OF + 1.12 2'FL	4.530	Restricted	285-325 ml MR/kg BW	PND 2-33
BMOS	Bovine derived milk oligosaccharides	5.79 BMOS	5.790	Restricted	285-325 ml MR/kg BW	PND 2-33
HMO	2'Fucosyllactose + Lacto-N-neotetraose	0.81 2'FL + 0.42 LNnT	1.230	Restricted	285-325 ml MR/kg BW	PND 2-33
BMOS + HMO	BMOS + HMO	5.75 BMOS + 1.00 2'FL + 0.53 LNnT	7.280	Restricted	285-325 ml MR/kg BW	PND 2-33

* Abbreviations: OS, oligosaccharide; MR, milk replacer; PND, postnatal day.
† Milk was reconsistuted at a rate of 200g of milk replacer per 1 liter of water.

Table 7.2: Analyzed nutrient composition of base formula

Nutrient	Units	Per kg*	Per Liter†
Energy and Macronutrients			
Total calories	kcal	5245	1049
Carbohydrate	g	285	57
Fat	g	320	64
Protein	g	305	61
Minerals			
Calcium	mg	11165	2233
Chlorine	mg	5705	1141
Copper	μg	8200	1640
Iodine	μg	1370	274
Iron	mg	95	19
Magnesium	mg	1135	227
Manganese	μg	11525	2305
Phosphorus	mg	8105	1621
Potassium	mg	11275	2255
Selenium	μg	325	65
Sodium	mg	8540	1708
Zinc	mg	85	17
Vitamins and other nutrients			
Vitamin A	IU	22860	4572
Vitamin D3	IU	3805	761
Vitamin E	IU	150	30
Vitamin K	μg	1605	321
Thiamin	μg	6610	1322
Riboflavin	μg	13040	2608
Niacin	μg	66830	13366
Vitamin B6	μg	6050	1210
Folic Acid	μg	1055	211
Vitamin B12	μg	30	6
Pantothenic Acid	μg	46080	9216
Biotin	μg	370	74
Choline	mg	1760	352
Arachidonic Acid	mg	1590	318
Docosahexaenoic Acid	mg	775	155
Sialyllactose	mg	290	58

* Diets were produced by Mead Johnson Nutrition (Evansville, IN, USA) using a proprietary blend of nutrients formulated to meet the nutritional needs of growing pigs. This formulation was used as the base for CON.PDX-GOS, PDX-GOS, CON.SL, and PDX-GOS + SL diets.

† Milk was reconstituted at a rate of 200g of milk replacer per 1 liter of water.

Table 7.3: Formulated nutrient composition of base formula

Nutrient	Units	Per kg*	Per Liter†
Energy and Macronutrients			
Metabolisable Energy	kcal	4286	857
Crude Protein	g	241	48
Crude Fat	g	241	48
Lactose	g	369	74
Crude Fiber	mg	20	4
Ash	g	85	17
Minerals			
Calcium	mg	10000	2000
Copper	mg	12	2
Total Phosphorous	mg	8000	1600
Potassium	mg	1835	367
Selenium	μg	345	69
Sodium	mg	8750	1750
Zinc	mg	120	24
Vitamins and other nutrients			
Vitamin A	IU	82427	16485
Vitamin D	IU	11564	2313
Vitamin E	IU	253	51
Lysine	g	25	5
Methione + Cysteine	g	10	2

* ProNurse® Specialty Milk Replacer (Purina Animal Nutrition, Gray Summit, MO) was used as the base formula and nutritional composition is adapted from advertised nutritent composition. This formulation was used as the base for CON.MO, BMOS, HMO, BMOS + HMO, OF, and OF + 2'FL diets.

† Milk was reconstituted at a rate of 200g of milk replacer per 1 liter of water.

Table 7.4: Body weight and milk intake regressed on the RI

Variable*†	Parm	CON.PDX-GOS	PDX-GOS	CON.SL	PDX-GOS + SL	CON.MO	BMOS	HMO	BMOS + HMO	OF	OF + 2'FL
Mean study body weight, kg	n	11	12	17	16	11	11	12	11	12	11
	b_1	-0.030	0.003	0.045	0.124	0.071	0.039	0.003	0.135	-0.151	-0.127
	SE	0.049	0.024	0.139	0.178	0.105	0.104	0.106	0.119	0.178	0.060
	R^2	0.042	0.002	0.007	0.033	0.048	0.015	0.000	0.124	0.068	0.334
	p	0.547	0.904	0.753	0.498	0.516	0.716	0.981	0.289	0.414	0.063
Mean daily milk intake, l	n	11	12	17	16	11	11	12	11	12	11
	b_1	-0.094	0.009	-0.273	-0.167	0.219	0.119	0.008	0.412	-0.464	-0.392
	SE	0.150	0.074	0.424	0.205	0.326	0.320	0.328	0.369	0.549	0.185
	R^2	0.042	0.002	0.027	0.045	0.048	0.015	0.000	0.122	0.067	0.334
	p	0.548	0.904	0.529	0.428	0.518	0.719	0.980	0.293	0.417	0.063

* All variables were regressed against the standardized Recognition Index individually.

† For all diets where milk was provided via restricted feeding, milk intake is a function of body weight, thus regression output is similar for both body weight and milk intake. Otherwise for diets where milk was provided freely (CON.SL and PDX-GOS + SL), milk intake is not a direct function of body weight.

Table 7.5: VFA regressed on the RI in the cecum

Variable*†	Parm	CON.PDX-GOS	PDX-GOS
DM	n	11	12
	b_1	0.068	-0.566
	SE	0.333	0.261
	R^2	0.005	0.320
	p	0.843	0.055
Ac	n	11	12
	b_1	-0.023	0.304
	SE	0.333	0.301
	R^2	0.001	0.092
	p	0.946	0.337
Pr	n	11	12
	b_1	0.014	0.431
	SE	0.333	0.285
	R^2	0.000	0.185
	p	0.968	0.162
Bu	n	11	12
	b_1	0.210	0.040
	SE	0.326	0.316
	R^2	0.044	0.002
	p	0.536	0.901
Iv	n	11	12
	b_1	-0.245	0.168
	SE	0.323	0.312
	R^2	0.060	0.028
	p	0.468	0.602
Va	n	11	12
	b_1	0.095	0.357
	SE	0.332	0.295
	R^2	0.009	0.127
	p	0.782	0.255
Ib	n	11	12
	b_1	-0.419	0.267
	SE	0.303	0.305
	R^2	0.176	0.071
	p	0.200	0.402

* All variables were standardized and regressed against the standardized Recognition Index individually.

† Abbreviations: Ac, acetate; Pr, propionate; Bu, butyrate; Iv, isovalerate; Va, valerate; Ib, isobutyrate.

Table 7.6: VFA regressed on the RI in the ascending colon

Variable*†	Parm	CON.PDX-GOS	PDX-GOS	CON.SL	PDX-GOS + SL	CON.MO	BMOS	HMO	BMOS + HMO	OF	OF + 2'FL
	n	11	12	17	16	8	7	8	6	8	7
	b_1	0.458	-0.265	0.083	-0.366	0.641	-0.379	-0.333	0.853	0.764	-0.670
DM	SE	0.296	0.305	0.257	0.262	0.313	0.378	0.385	0.294	0.263	0.477
	R^2	0.209	0.070	0.007	0.122	0.411	0.167	0.111	0.678	0.584	0.283
	p	0.157	0.405	0.752	0.184	0.087	0.362	0.421	0.044	0.027	0.219
	n	11	12	17	16	8	7	8	6	8	7
	b_1	0.066	-0.208	0.082	0.063	-0.291	0.476	0.044	-0.171	-0.718	0.958
Ac	SE	0.333	0.309	0.257	0.310	0.391	0.358	0.408	0.511	0.284	0.539
	R^2	0.004	0.043	0.007	0.003	0.085	0.261	0.002	0.027	0.516	0.387
	p	0.848	0.516	0.754	0.843	0.484	0.241	0.918	0.754	0.045	0.136
	n	11	12	17	16	8	7	8	6	8	7
	b_1	0.340	-0.230	0.150	0.141	-0.184	0.505	0.175	0.102	-0.791	0.681
Pr	SE	0.314	0.308	0.255	0.274	0.401	0.350	0.402	0.515	0.250	0.363
	R^2	0.115	0.053	0.023	0.019	0.034	0.294	0.031	0.010	0.626	0.414
	p	0.307	0.472	0.565	0.615	0.663	0.208	0.679	0.853	0.019	0.119
	n	11	12	17	16	8	7	8	6	8	7
	b_1	0.426	-0.719	-0.338	-0.244	-0.205	0.605	0.247	0.353	0.333	0.637
Bu	SE	0.302	0.220	0.243	0.267	0.400	0.314	0.396	0.487	0.385	0.371
	R^2	0.181	0.517	0.114	0.056	0.042	0.427	0.061	0.116	0.111	0.371
	p	0.192	0.008	0.185	0.376	0.626	0.112	0.555	0.508	0.421	0.147
	n	11	12	17	16	8	7	8	6	8	7
	b_1	0.055	-0.606	-0.538	0.155	0.325	0.503	0.030	-0.853	0.339	0.288
Iv	SE	0.333	0.252	0.218	0.262	0.386	0.349	0.408	0.294	0.384	0.433
	R^2	0.003	0.367	0.290	0.024	0.106	0.293	0.001	0.678	0.115	0.081
	p	0.873	0.037	0.026	0.563	0.432	0.210	0.944	0.044	0.412	0.536
	n	11	12	17	16	8	7	8	6	8	7
	b_1	0.410	-0.439	-0.306	-0.041	0.022	0.225	-0.123	0.298	-0.487	0.585
Va	SE	0.304	0.284	0.246	0.265	0.408	0.402	0.405	0.496	0.357	0.321
	R^2	0.168	0.193	0.094	0.002	0.001	0.059	0.015	0.083	0.237	0.400
	p	0.210	0.153	0.232	0.880	0.958	0.599	0.771	0.580	0.222	0.128
	n	11	12	17	16	8	7	8	6	8	7
	b_1	-0.092	-0.627	-0.490	0.257	-0.225	0.169	-0.087	0.404	-0.555	0.604
Ib	SE	0.332	0.246	0.225	0.276	0.398	0.416	0.407	0.477	0.340	0.331
	R^2	0.008	0.393	0.240	0.058	0.050	0.032	0.008	0.152	0.308	0.400
	p	0.789	0.029	0.046	0.367	0.593	0.701	0.838	0.445	0.154	0.128

* All variables were standardized and regressed against the standardized Recognition Index individually.
† Abbreviations: Ac, acetate; Pr, propionate; Bu, butyrate; Iv, isovalerate; Va, valerate; Ib, isobutyrate.

Table 7.7: VFA regressed on the RI in feces

Variable*†	Parm	CON.PDX-GOS	PDX-GOS	CON.SL	PDX-GOS + SL
DM	n	10	12	17	16
	b_1	-0.168	0.650	0.144	-0.566
	SE	0.327	0.240	0.256	0.211
	R^2	0.032	0.422	0.021	0.339
	p	0.621	0.022	0.581	0.018
Ac	n	10	12	17	16
	b_1	0.413	-0.299	0.207	0.257
	SE	0.299	0.302	0.253	0.250
	R^2	0.193	0.089	0.043	0.070
	p	0.204	0.346	0.424	0.321
Pr	n	10	12	17	16
	b_1	0.347	0.083	0.098	0.316
	SE	0.309	0.315	0.257	0.245
	R^2	0.136	0.007	0.010	0.106
	p	0.294	0.799	0.707	0.217
Bu	n	10	12	17	16
	b_1	-0.164	-0.356	0.262	0.235
	SE	0.327	0.295	0.249	0.257
	R^2	0.030	0.127	0.069	0.056
	p	0.631	0.256	0.309	0.376
Iv	n	10	12	17	16
	b_1	0.142	-0.143	0.156	0.224
	SE	0.329	0.313	0.255	0.252
	R^2	0.023	0.020	0.024	0.053
	p	0.678	0.658	0.550	0.391
Va	n	10	12	17	16
	b_1	-0.356	-0.020	0.096	0.073
	SE	0.308	0.316	0.257	0.270
	R^2	0.143	0.000	0.009	0.005
	p	0.281	0.951	0.715	0.790
Ib	n	10	12	17	16
	b_1	0.078	-0.142	0.047	0.323
	SE	0.331	0.313	0.258	0.244
	R^2	0.007	0.020	0.002	0.111
	p	0.819	0.660	0.856	0.207

* All variables were standardized and regressed against the standardized Recognition Index individually.

† Abbreviations: Ac, acetate; Pr, propionate; Bu, butyrate; Iv, isovalerate; Va, valerate; Ib, isobutyrate.

Table 7.8: Predictors of Recognition Index with outliers

	Dependent variable:							
	RI							
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Iv	-0.029^{**}					-0.036^{*}		
	(0.012)					(0.017)		
Ib		-0.042^{**}					-0.055^{**}	
		(0.016)					(0.024)	
Bu			-0.010^{***}					-0.012^{***}
			(0.003)					(0.004)
Striatal_Ser				-0.815		0.610	0.776	
				(0.831)		(0.978)	(0.969)	
Striatal_Epi					-0.003			0.005
					(0.004)			(0.004)
Constant	0.873^{***}	0.884^{***}	0.907^{***}	0.856^{***}	0.769^{***}	0.806^{***}	0.802^{***}	0.864^{***}
	(0.070)	(0.071)	(0.063)	(0.148)	(0.092)	(0.129)	(0.125)	(0.069)
Observations	12	12	12	12	12	12	12	12
R^2	0.367	0.393	0.517	0.088	0.038	0.394	0.433	0.592
Adjusted R^2	0.304	0.332	0.469	-0.004	-0.058	0.259	0.307	0.501
Akaike Inf. Crit.	-21.665	-22.153	-24.912	-17.271	-16.637	-20.172	-20.981	-24.922
Bayesian Inf. Crit.	-20.210	-20.698	-23.458	-15.817	-15.182	-18.232	-19.041	-22.983

() indicates standard error *p<0.1; **p<0.05; ***p<0.01

Table 7.9: Predictors of Recognition Index without outliers

	Dependent variable:							
	RI							
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Iv	-0.085^{**}					-0.086^{**}		
	(0.029)					(0.031)		
Ib		-0.092^{**}					-0.097^{**}	
		(0.035)					(0.038)	
Bu			-0.009^{**}					-0.013^{**}
			(0.003)					(0.005)
Striatal_Ser				-0.076		0.233	0.498	
				(1.170)		(0.892)	(0.946)	
Striatal_Epi					-0.003			0.005
					(0.004)			(0.004)
Constant	1.145^{***}	1.063^{***}	0.898^{***}	0.738^{***}	0.784^{***}	1.111^{***}	0.997^{***}	0.866^{***}
	(0.145)	(0.131)	(0.070)	(0.198)	(0.089)	(0.201)	(0.185)	(0.074)
Observations	11	11	11	11	11	11	11	11
R^2	0.488	0.433	0.431	0.0005	0.052	0.492	0.452	0.513
Adjusted R^2	0.431	0.370	0.368	-0.111	-0.054	0.365	0.315	0.392
Akaike Inf. Crit.	-22.691	-21.567	-21.532	-15.332	-15.909	-20.785	-19.941	-21.252
Bayesian Inf. Crit.	-21.497	-20.374	-20.339	-14.138	-14.715	-19.193	-18.350	-19.661

() indicates standard error *p<0.1; **p<0.05; ***p<0.01

Table 7.10: Serotonin does not mediate the relationship between RI and Iv

Parameter*	Estimate	95% Lower CI	95% Upper CI	*p*-value
ACME	0.0072	-0.0194	0.05	0.476
ADE	-0.0361	-0.1465	-0.02	0.021
Total Effect	-0.0289	-0.1309	-0.02	0.001
Prop. Mediated	-0.2499	-1.6479	0.45	0.475

* Abbreviations: RI, recognition index; Iv, isovalerate; ACME, average casual mediation effect; ADE, average direct effect; Prop. Mediated, Proportion mediated (ACME/Total Effect); CI, confidence interval.

Table 7.11: Epinephrine does not mediate the relationship between RI and Bu

Parameter*	Estimate	95% Lower CI	95% Upper CI	*p*-value
ACME	0.0026	-0.0035	0.01	0.349
ADE	-0.0123	-0.0194	-0.01	0.020
Total Effect	-0.0097	-0.0143	0.00	0.003
Prop. Mediated	-0.2703	-1.8136	0.37	0.350

* Abbreviations: RI, recognition index; Iv, isovalerate; ACME, average casual mediation effect; ADE, average direct effect; Prop. Mediated, Proportion mediated (ACME/Total Effect); CI, confidence interval.

7.9 Figures

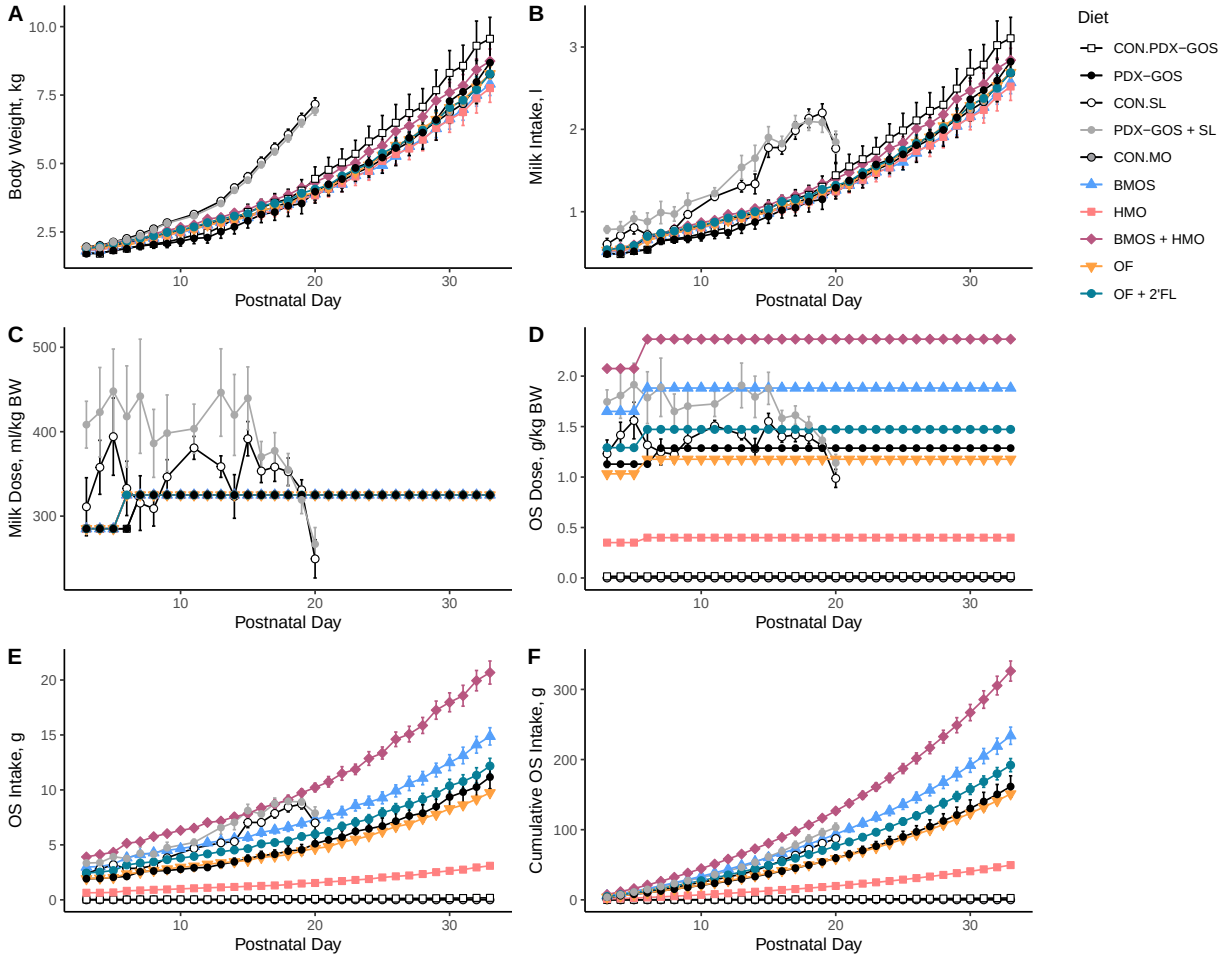

Figure 7.1: Growth, milk intake, and oligosaccharide intake across all diets. Note Postnatal Days 10 and 12 are absent from groups CON.SL and PDX-GOS + SL due to an error in data collection. Abbreviations: OS, oligosaccharide.

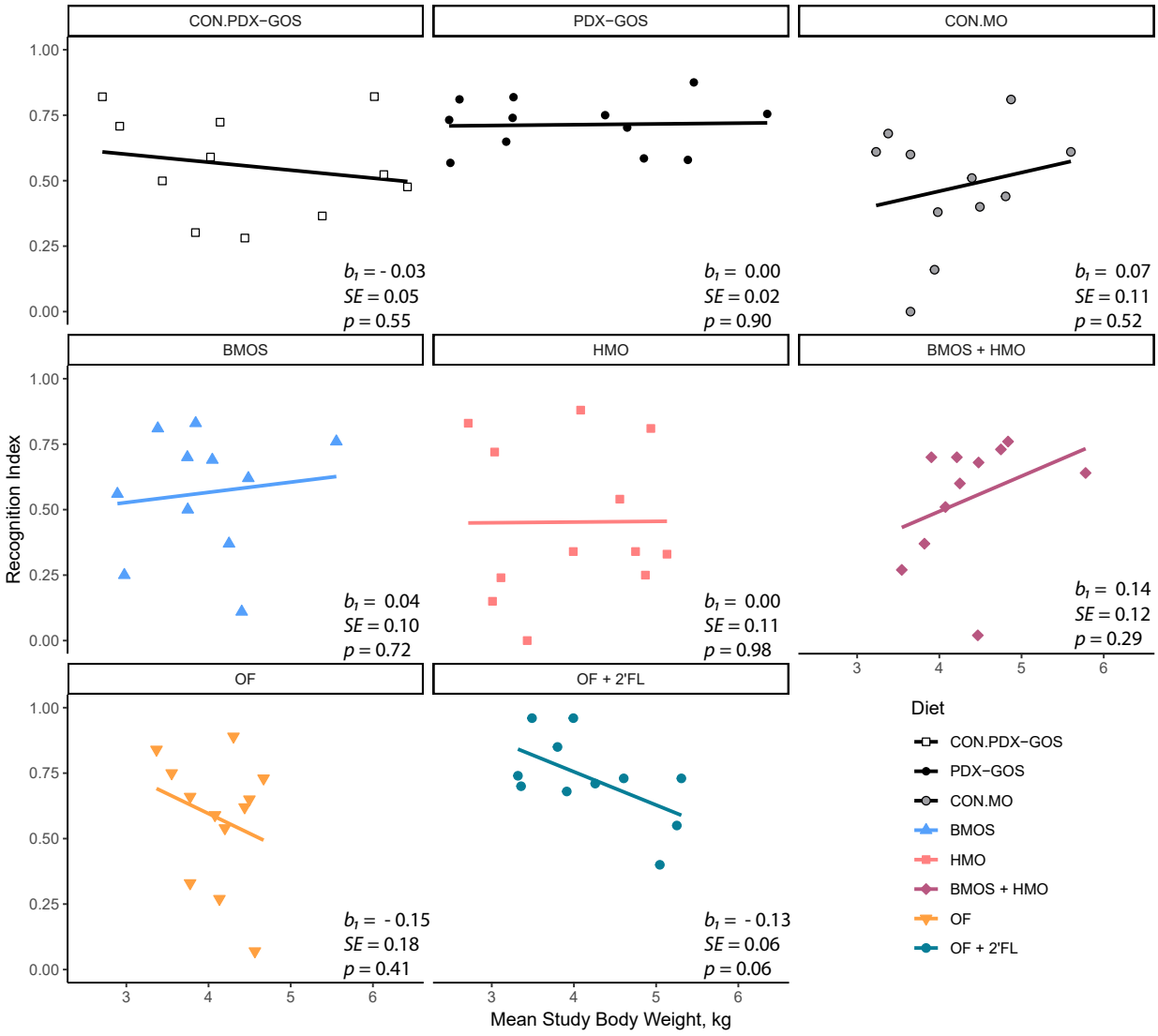

Figure 7.2: Mean study body weight regressed against the recognition index for all diets where milk was provided via restricted feeding. Here, milk and oligosaccharide intake is a function of body weight, thus regression output for those variables are similar to body weight and not shown.

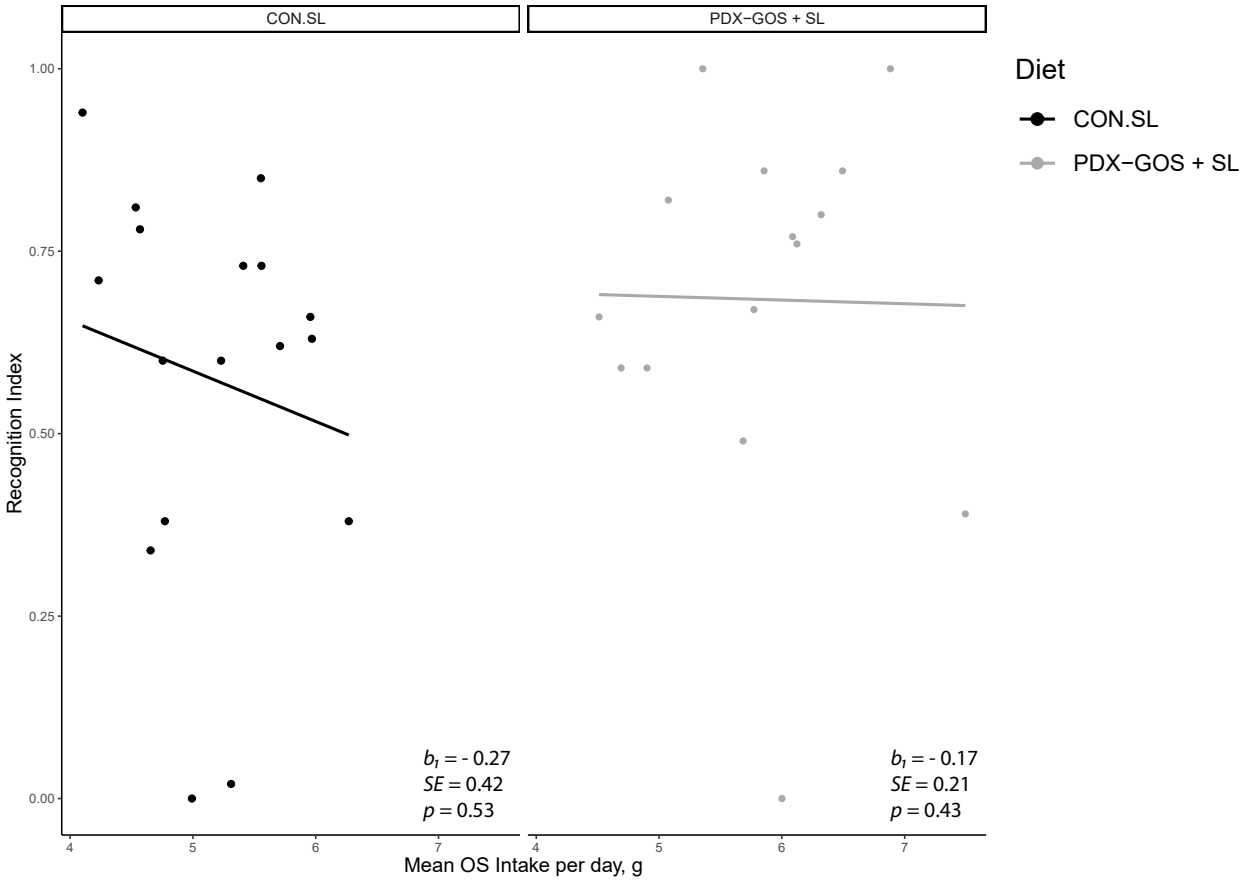

Figure 7.3: Mean oligosaccharide (OS) intake per day regressed against the recognition index for all diets where milk was provided *ad libitum*. Here, oligosaccharide intake is a direct function of milk intake, however milk intake was not a direct function of body weight..

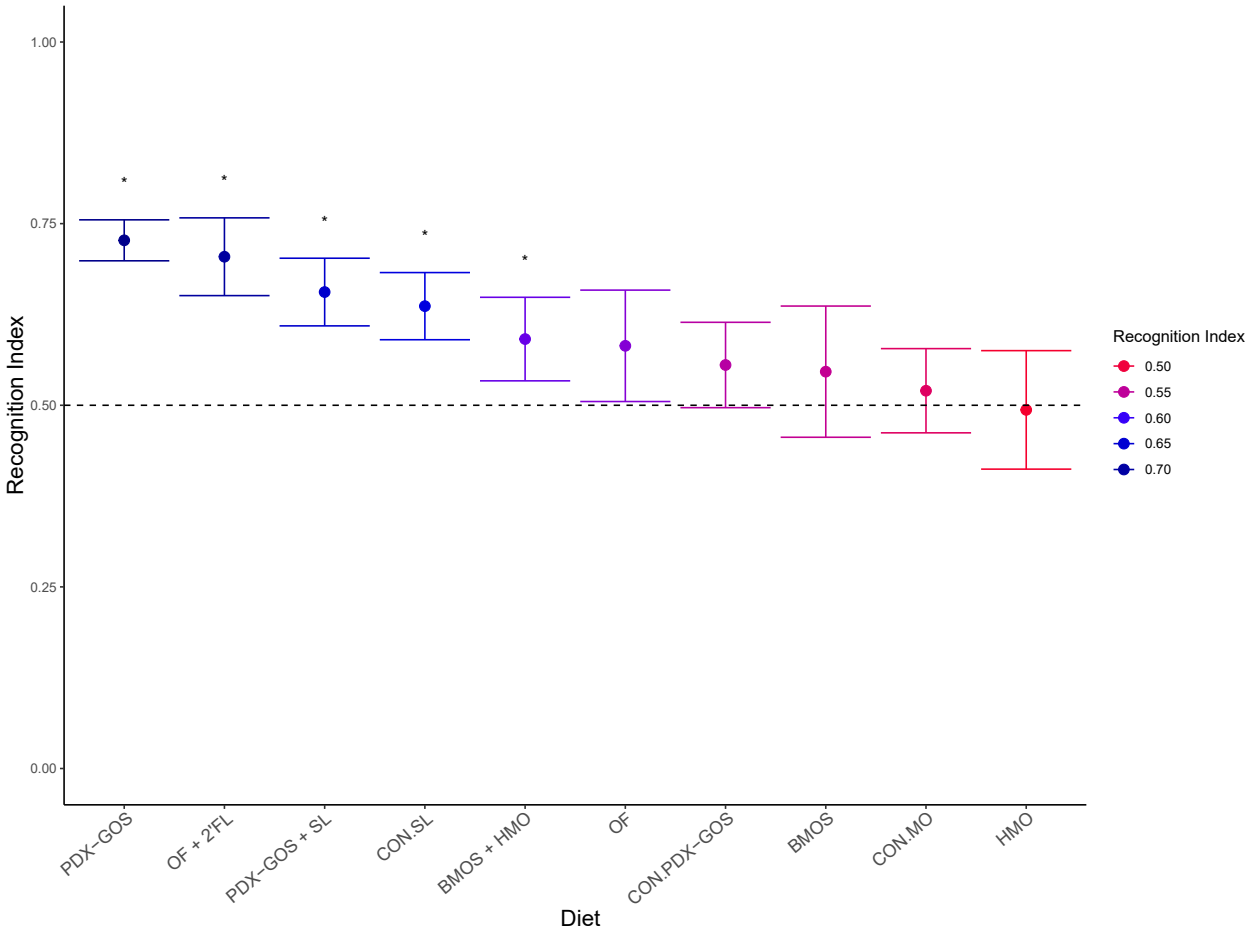

Figure 7.4: Average recognition index scores across all diets tested. Asterisk indicates performances significantly above 0.50.

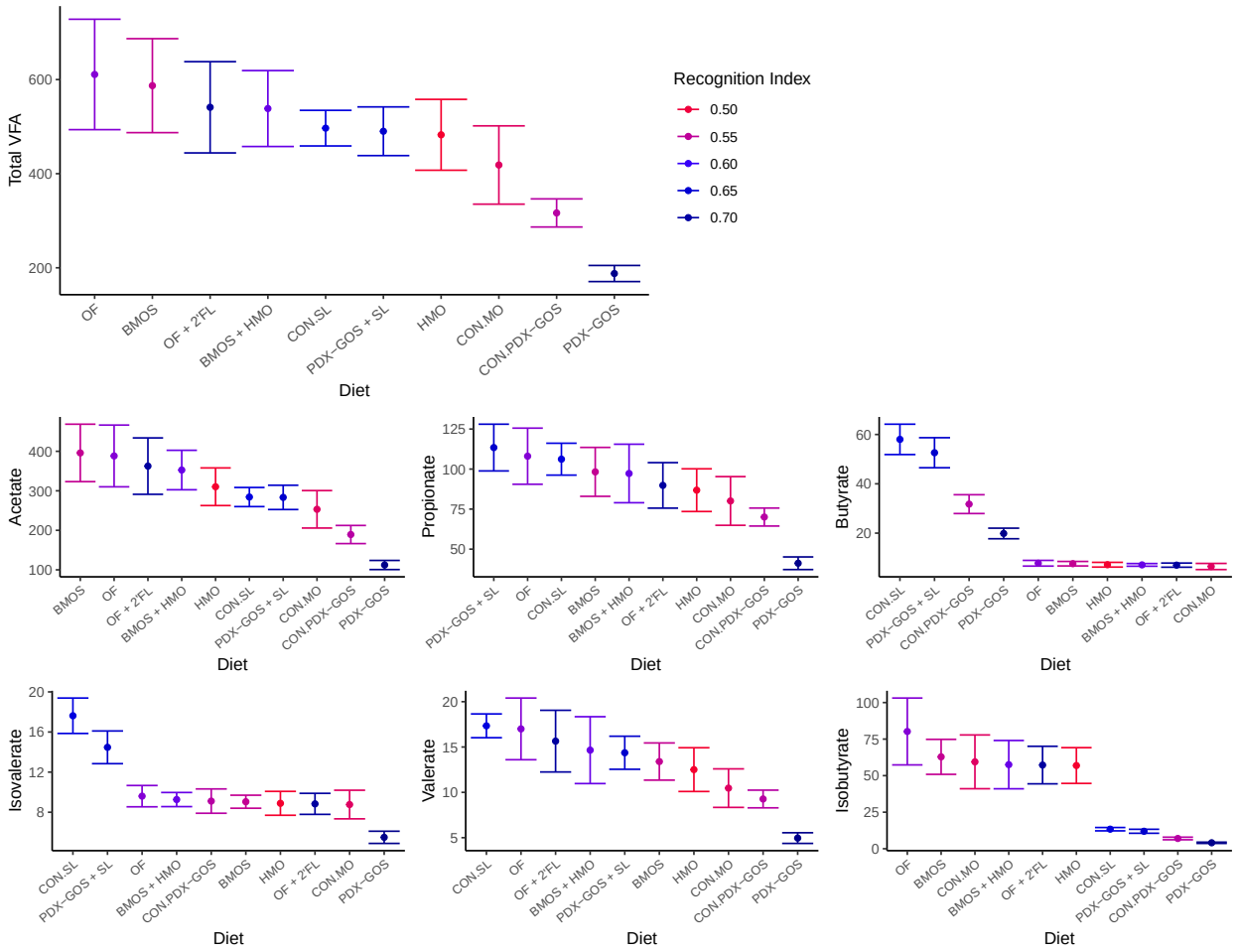

Figure 7.5: Concentration of volatile fatty acids (VFA) in the ascending colon. Values are expressed as μmol/g dry matter. Color indicates performance on the novel object recognition task. There does not appear to be a clear relationship between recognition index and VFA concentration between diets.

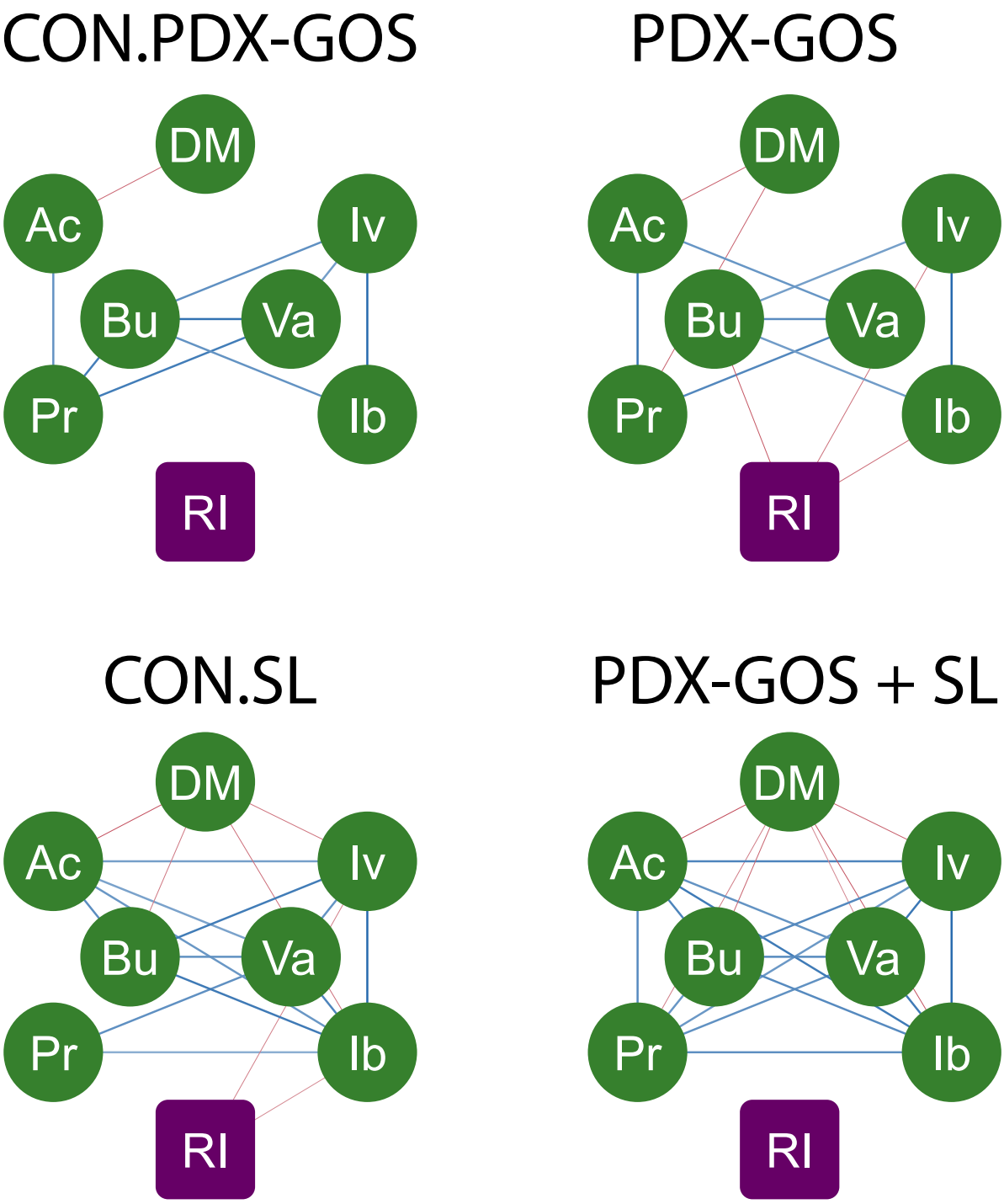

Figure 7.6: Correlation network expressing the relationship between volatile fatty acids in the ascending colon and the recognition index (RI) from studies investigating PDX-GOS (Chapters 3 and 4). Only significant relationships are shown ($p < 0.05$), with thicker lines indicating smaller p-values and blue/red indicating a positive/negative relationship. Abbreviations: DM, dry matter; Ac, acetate; Bu, butyrate; Pr, propionate; Iv, isovalerate; Va, valerate; Ib, isobutyrate.

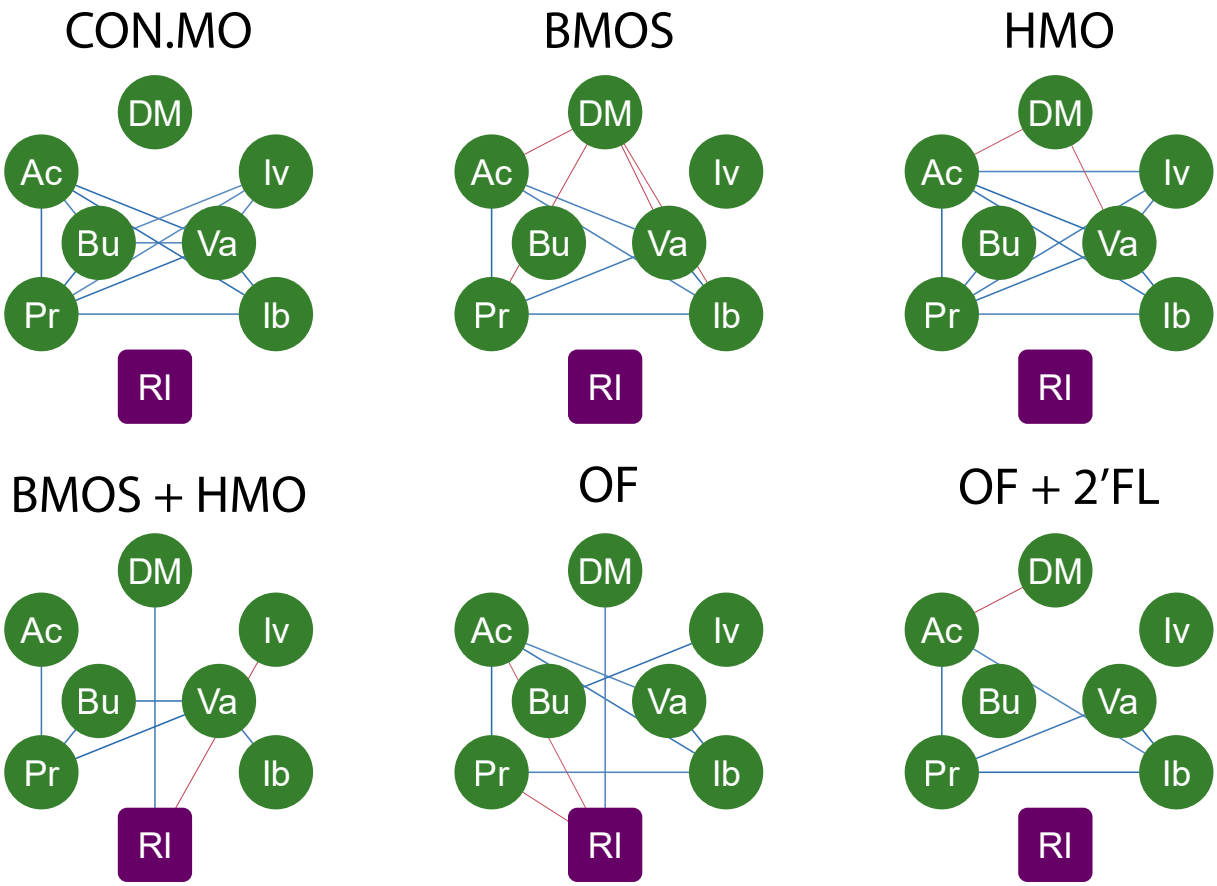

Figure 7.7: Correlation network expressing the relationship between volatile fatty acids in the ascending colon and the recognition index (RI) from studies investigating milk oligosaccharides (Chapters 5 and 6). Only significant relationships are shown ($p < 0.05$), with thicker lines indicating smaller p-values and blue/red indicating a positive/negative relationship. Abbreviations: DM, dry matter; Ac, acetate; Bu, butyrate; Pr, propionate; Iv, isovalerate; Va, valerate; Ib, isobutyrate.

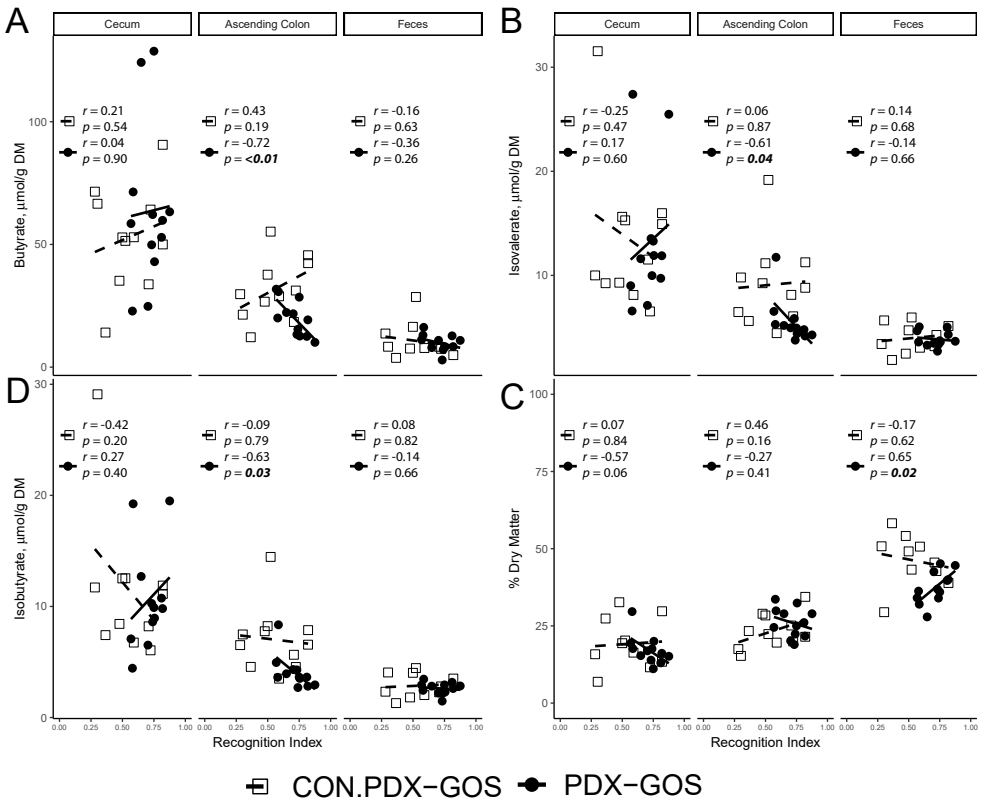

Figure 7.8: Correlations between select VFA known to be related to the recognition index separated by region of the colon and diet. As depicted, VFA in the cecum and feces are unrelated to the recognition index, however percent dry matter in the feces positively predicted recognition index in pigs fed PDX-GOS. Data adapted from Chapter 3.

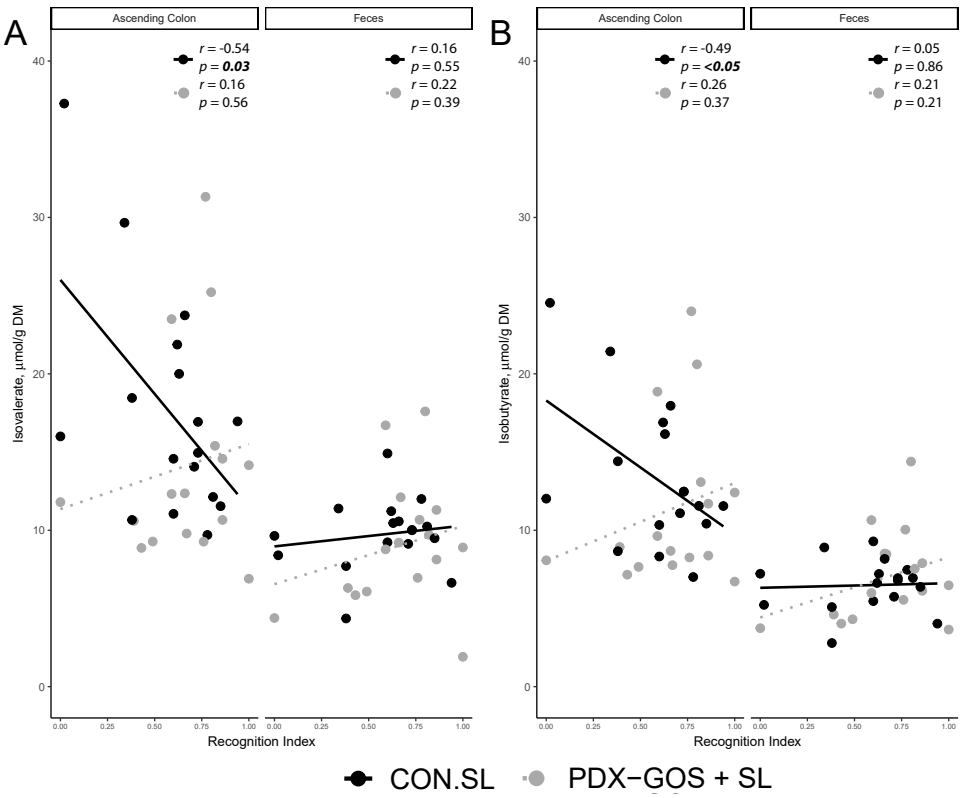

Figure 7.9: Correlations between select VFA known to be related to the recognition index separated by region of the colon and diet. As depicted, both isovalerate and isobutyrate in the ascending colon, but not feces, are inversely related to the recognition index in pigs fed the CON.SL diet (which contained PDX-GOS). Data adapted from Chapter 4.

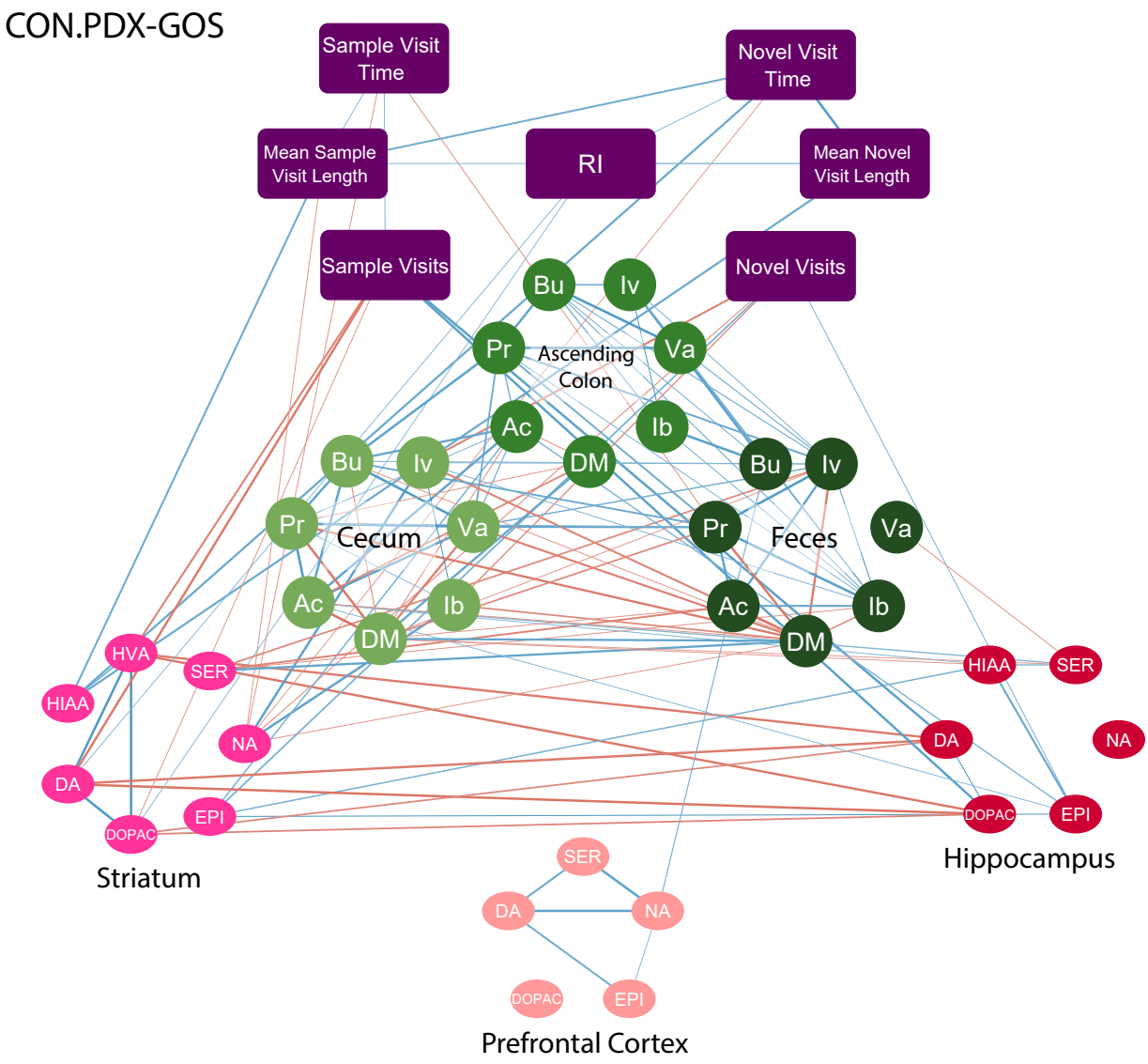

Figure 7.10: Correlation network from the CON.PDX-GOS group adapted from Chapter 3. Although some VFA are related to measures of exploratory behavior, no volatile fatty acids were related to the recognition index. However dopamine and it's metabolic DOPAC were positvely related to the recognition index. Only significant relationships are shown ($p < 0.05$), with thicker lines indicating smaller p-values and blue/red indicating a positive/negative relationship. Abbreviations: Ac, acetate; Bu, butyrate; DA, dopamine; DM, dry matter; DOPAC, 3,4-dihydroxyphenylacetic acid; EPI, epinephrine; HIAA, 5'hydroxyindoleacetic acid; HVA, homovanillica acid; Ib, isobutyrate; Iv, isovalerate; NA, noreprinephrine; Pr, propionate; RI, recognition index; SER, serotonin; Va, valerate.

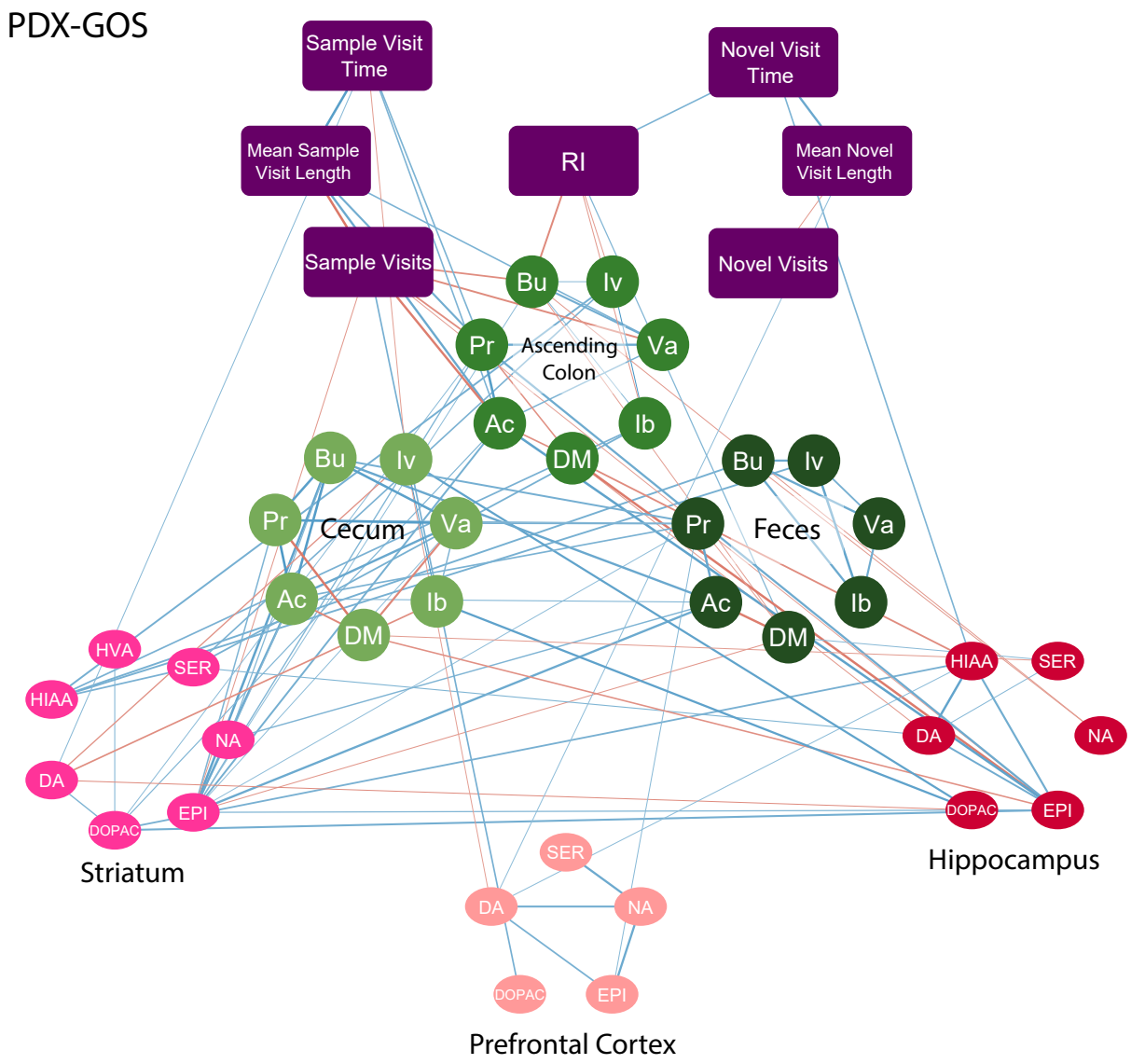

Figure 7.11: Correlation network from the PDX-GOS group adapted from Chapter 3. As shown previously, the VFA butyrate, isovalerate, and isobutyrate in the ascending colon relate to the recognition index. Although some relationships between VFA and catecholamines exist, no catecholamines were related to the recognition index. Only significant relationships are shown ($p < 0.05$), with thicker lines indicating smaller p-values and blue/red indicating a positive/negative relationship. Abbreviations: Ac, acetate; Bu, butyrate; DA, dopamine; DM, dry matter; DOPAC, 3,4-dihydroxyphenylacetic acid; EPI, epinephrine; HIAA, 5'hydroxyindoleacetic acid; HVA, homovanillica acid; Ib, isobutyrate; Iv, isovalerate; NA, noreprinephrine; Pr, propionate; RI, recognition index; SER, serotonin; Va, valerate.

Chapter 8

Conclusions

The aim of this dissertation was to evaluate the evidence supporting the role of oligosaccharides in promoting cognitive development. Through the use of the pig as a novel animal model for studying nutritional neuroscience, we performed several experiments assessing how the inclusion of indigestible and fermentable oligosaccharides in the early-life diet impact recognition memory. We chose to measure recognition memory in each study due to the ease and high throughput manner with which the novel object recognition task be administered. Furthermore, the paradigm that task is based on is analogous to the visual paired comparison task used in infants. In a series of experiments, we investigated polydextrose (PDX) and galactooligosaccharide (GOS), sialyllactose (SL), oligofructose (OF), bovine milk oligosaccharides (BMOS), and 2'fucosyllactose and lacto-N-neotetraose (HMO). We found that several of these oligosaccharides, sometimes alone or in combination with others, were capable of improving recognition memory as compared to control subjects not receiving any oligosaccharides in the diet. Our findings build upon those in rodent and other pig research demonstrating that oligosaccharides have beneficial properties with relation to cognition. Common to the inquiry of most scientific endeavors, the generalization of these findings is difficult due to the various dosages, feeding regimens, and experimental designs used across studies. Why some oligosaccharides and not others improve cognition is still to be discovered, however there is mounting evidence suggesting they do play a significant role in cognitive development.

8.1 Validation of the novel object recognition task in pigs

To use the pig to study cognitive development it's capabilities must first be established. We assessed the effects of sex, age, and delay length on the performance of young pigs in the novel object recognition task. Our data suggest that pigs were able to discriminate between novel and sample objects after delays of 2 min, 1 h, 24 h, and 48 h, but recognition of the novel object was dependent on factors assessed. Females were able to display novelty preferences at both the 1- and 2-day delays, whereas males were unable to exhibit

novelty preferences in the 24-h delay. Sex and age played a significant role in the NOR task, as females and older pigs tended to exhibit greater exploratory behavior than males and younger pigs. Such performance, however, did not extend towards the novel location recognition task, as pigs were only able to discriminate between novel and sample location on the shortest delay tested. These data provided evidence that the NOR task could be used in pigs and would likely be sensitive enough to measure differences in development due to nutrition. As nutritive effects on brain development are sometimes small and hard to observe, there was a need to develop a task that would be sensitive enough yet easy to conduct for identifying these small differences.

8.2 Polydextrose and Galactooligosaccharide

Both polydextrose and galactooligosaccharide are added to some infant formulas due to their known capability to alter fecal consistency in infants. Here we assessed if PDX and GOS in early life could affect behavior. We found pigs fed this combination performed significantly better on the NOR task than control pigs, had lower hippocampal serotonin, and lower colonic VFA. In a follow-up study we assessed if the addition of sialyllactose could further improve behavioral performance and found no additional benefit as assessed by the NOR task and diurnal activity. We failed to replicate the effects of this prebiotic on anxiety-like behavior through the use of the back-test, and additionally did not find an alteration in expression of the learning related proteins PSD-95, CREB, and synaptophysin. Ultimately these two studies provided strong evidence that PDX and GOS promote the development of recognition memory in early life.

8.3 Milk Oligosaccharides

In a 2-part study we investigated the impact of OF, HMO, and BMOS on recognition memory, structural brain development, and hippocampal gene expression. Using both a short and long delay on the NOR task demonstrated that these oligosaccharides acted differentially on recognition memory when provided alone or in combination. Individual oligosaccharides were capable of improving short-term memory but only combinations of oligosaccharides improved long-term memory. Oligofructose in general had little impact on structural brain development, whereas both bovine and human milk oligosaccharides altered the volume of both cortical and subcortical structures. No oligosaccharides had large impacts on neurochemistry as measured by magnetic resonance spectroscopy or on myelination as measured by diffusion tensor imaging. Hippocampal gene expression showed a handful of genes were impacted by the diet; however, the functional consequences of these changes are unclear. Several genes were related to performance on the novel object recognition task, but these relationships differed by diet. Despite the mixed results, we found strong evidence that some combinations of milk oligosaccharides do impact brain development, and significant research is required to further the replicability and mechanisms of these effects.

8.4 Volatile Fatty Acids and Recognition Memory

To explore possible mechanistic links within the gut-brain-axis we investigated whether volatile fatty acids in the colon relate to recognition memory. We found several relationships between both short and branched chain fatty acids and recognition memory, but these tended to be most replicable with branched chain fatty acids. These relationships were not equally present across different diets, but we found with the repeated investigation of polydextrose and galactooligosaccharide that isovalerate and isobutyrate negatively related to recognition memory. Importantly, this was a diet-dependent effect that only occurred with volatile fatty acids in the ascending colon, not in the cecum or the feces. Additionally, we found that catecholamines did not mediate this effect, as serotonin, dopamine, and their metabolites in a few key brain regions had no relationship with recognition memory.

8.5 Limitations

While the benefits of using a piglet model have been described, the limitations of the pig in addition to infant research in nutrition and brain development warrant attention. In the investigation of the impact of HMO on cognition, it is nearly impossible to define "normal" intake. As the composition of oligosaccharides in human milk is highly variable both in concentration and variety. Thus, the establishment of normative values to test in animal research is not possible. Furthermore, as no other animal contains such a vast diversity and high concentration of HMO, any species that HMO could be tested with likely did not evolve with the same gastrointestinal and microbial mechanisms with which to digest HMO. While most data in animal research suggests at worst neutral effects on host physiology, the translatability of animal research with HMO may be questioned. Though animal research suffers from translatability, the use of validated, accurate, and domain specific behavioral tasks are readily at hand in animal research. It is a prohibitively large task to use specific and laborious cognitive tasks for assessment of infant cognition in large clinical trials. General intelligence, verbal, and motor tasks used for cognitive assessment are tools that are ill-suited to describing specific and subtle effects of nutritional interventions on cognitive development, potentially leading to the discrepancies observed between various studies.

8.6 Summary

As covered in depth in this thesis, there is significant and mounting evidence that dietary oligosaccharides contribute to cognitive development. Here we show the replicability of some oligosaccharides while highlighting the differential nature of others in promoting recognition memory. There is no clear pattern based on dosage or structure to indicate why some oligosaccharides have greater potential than others. However, we demonstrate that the concentration of volatile fatty acids in the colon relates to recognition memory in a diet-dependent manner, suggesting a possible route of action for their impact.

References

Albarracín, M., Weisstaub, A. R., Zuleta, A., Mandalunis, P., González, R. J., Drago, S. R., Albarracín, M., Weisstaub, A. R., Zuleta, Á., Mandalunis, P., González, R. J., and Drago, S. R. (2014). Effects of extruded whole maize, polydextrose and cellulose as sources of fibre on calcium bioavailability and metabolic parameters of growing Wistar rats. *Food and Function*, 5(4):804–810.

Aldredge, D. L., Geronimo, M. R., Hua, S., Nwosu, C. C., Lebrilla, C. B., and Barile, D. (2013). Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. *Glycobiology*, 23(6):664–676.

Altschuler, S. M., Escardo, J., Lynn, R. B., and Miselis, R. R. (1993). The Central Organization of the Vagus Nerve Colon of the Rat Innervating the. *Gastroenterology*, 104:502–509.

Antunes, M. and Biala, G. (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive processing*, 13(2):93–110.

Arave, C. W. (1996). Assessing sensory capacity of animals using operant technology. *Journal of animal science*, 74:1996–2009.

Arslanoglu, S., Moro, G. E., Schmitt, J., Tandoi, L., Rizzardi, S., and Boehm, G. (2008). Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. *The Journal of nutrition*, 138(6):1091–1095.

Ashley, C., Johnston, W. H., Harris, C. L., Stolz, S. I., Wampler, J. L., and Berseth, C. L. (2012). Growth and tolerance of infants fed formula supplemented with polydextrose (PDX) and/or galactooligosaccharides (GOS): double-blind, randomized, controlled trial. *Nutrition journal*, 11(1):38.

Baeza, J., Smallegan, M. J., and Denu, J. M. (2016). Mechanisms and Dynamics of Protein Acetylation in Mitochondria. *Trends in Biochemical Sciences*, 41(3):231–244.

Baillargeon, R. and DeVos, J. (1991). Object permanence in young infants: further evidence. *Child development*, 62(6):1227–1246.

Baillargeon, R., Spelke, E. S., and Wasserman, S. (1985). Object permanence in five-month-old infants. *Cognition*, 20(3):191–208.

Balderas, I., Rodríguez-Ortiz, C. J., Salgado-Tonda, P., Chávez-Hurtado, J., McGaugh, J. L., and Bermúdez-Rattoni, F. (2008). The consolidation of object and context recognition memory involve different regions of the temporal lobe. *Learning & Memory*, 15(9):618–624.

Baldwin, B. (1976). Quantitative studies on taste preference in pigs. *Proceedings of the Nutrition Society*, 35:69–73.

Ballard, O. and Morrow, A. L. (2013). Human milk composition: nutrients and bioactive factors. *Pediatric clinics of North America*, 60(1):49–74.

Barbosa, F. F., Santos, J. R., Meurer, Y. S. R., Macêdo, P. T., Ferreira, L. M. S., Pontes, I. M. O., Ribeiro, A. M., and Silva, R. H. (2013). Differential Cortical c-Fos and Zif-268 Expression after Object and Spatial Memory Processing in a Standard or Episodic-Like Object Recognition Task. *Frontiers in behavioral neuroscience*, 7:112.

Barichello, T., Generoso, J. S., Simões, L. R., Faller, C. J., Ceretta, R. A., Petronilho, F., Lopes-Borges, J., Valvassori, S. S., and Quevedo, J. (2014). Sodium Butyrate Prevents Memory Impairment by Re-establishing BDNF and GDNF Expression in Experimental Pneumococcal Meningitis. *Molecular Neurobiology*, 52(1):734–740.

Barker, G. R. I. and Warburton, E. C. (2011). When Is the Hippocampus Involved in Recognition Memory? *Journal of Neuroscience*, 31(29):10721–10731.

Barr, R., Dowden, A., and Hayne, H. (1996). Developmental changes in deferred imitation by 6- to 24-month-old infants. *Infant Behavior and Development*, 19:159–170.

Baudry, M. (2001). Long-term Potentiation (Hippocampus). In *International Encyclopedia of the Social & Behavioral Sciences*, pages 9081–9083. Elsevier.

Belfort, M. B., Rifas-Shiman, S. L., Kleinman, K. P., Guthrie, L. B., Bellinger, D. C., Taveras, E. M., Gillman, M. W., and Oken, E. (2013). Infant Feeding and Childhood Cognition at Ages 3 and 7 Years. *JAMA Pediatrics*, 167(9):836.

Ben, X. M., Li, J., Feng, Z. T., Shi, S. Y., Lu, Y. D., Chen, R., and Zhou, X. Y. (2008). Low level of galacto-oligosaccharide in infant formula stimulates growth of intestinal Bifidobacteria and Lactobacilli. *World Journal of Gastroenterology*, 14(42):6564–6568.

Ben, X.-m., Zhou, X.-y., Zhao, W.-h., Yu, W.-l., Pan, W., Zhang, W.-l., Wu, S.-m., Van Beusekom, C. M., and Schaafsma, A. (2004). Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants. *Chinese medical journal*, 117(6):927–31.

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., Deng, Y., Blennerhassett, P., MacRi, J., McCoy, K. D., Verdu, E. F., and Collins, S. M. (2011). The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology*, 141(2):599–609.

Berding, K., Wang, M., Monaco, M. H., Alexander, L. S., Mudd, A. T., Chichlowski, M., Waworuntu, R. V., Berg, B. M., Miller, M. J., Dilger, R. N., and Donovan, S. M. (2016). Prebiotics and bioactive milk fractions affect gut development, microbiota, and neurotransmitter expression in piglets. *Journal of pediatric gastroenterology and nutrition*, 63(6):688–697.

Bertelsen, R. J., Jensen, E. T., and Ringel-Kulka, T. (2016). Use of probiotics and prebiotics in infant feeding. *Best Practice & Research in Clinical Gastroenterology*, 30(1):39–48.

Biggio, F., Gorini, G., Utzeri, C., Olla, P., Marrosu, F., Mocchetti, I., and Follesa, P. (2009). Chronic vagus nerve stimulation induces neuronal plasticity in the rat hippocampus. *The international journal of neuropsychopharmacology*, 12(9):1209–21.

Blair, R. and Fitzsimmons, J. (1970). A note on the voluntary feed intake and growth of pigs given diets containing an extremely bitter compound. *Animal Production*, 12:529–530.

Bode, L. (2012). Human milk oligosaccharides: Every baby needs a sugar mama. *Glycobiology*, 22(9):1147–1162.

Boler, B. M. V., Serao, M. C. R., Faber, T. a., Bauer, L. L., Chow, J., Murphy, M. R., and Fahey, G. C. (2013). In vitro fermentation characteristics of select nondigestible oligosaccharides by infant fecal inocula. *Journal of Agricultural and Food Chemistry*, 61:2109–2119.

Bolhuis, J. E. (2004). Personalities in pigs: Individual characteristics and coping with environmental challenges. page 176.

Bonthuis, P. J., Patteson, J. K., and Rissman, E. F. (2011). Acquisition of sexual receptivity: roles of chromatin acetylation, estrogen receptor-alpha, and ovarian hormones. *Endocrinology*, 152(8):3172–81.

Botting, N. P. (1995). Chemistry and Neurochemistry of the Kynurenine Pathway of Tryptophan Metabolism. *Chemical Society Reviews*, 24(6):401–412.

Bourassa, M. W., Alim, I., Bultman, S. J., and Ratan, R. R. (2016). Butyrate, neuroepigenetics and the gut microbiome: Can a high fiber diet improve brain health? *Neuroscience Letters*, 625:56–63.

Braniste, V., Al-Asmakh, M., Kowal, C., Anuar, F., Abbaspour, A., Toth, M., Korecka, A., Bakocevic, N., Ng, L. G., Kundu, P., Gulyas, B., Halldin, C., Hultenby, K., Nilsson, H., Hebert, H., Volpe, B. T., Diamond, B., and Pettersson, S. (2014). The gut microbiota influences blood-brain barrier permeability in mice. *Science Translational Medicine*, 6(263):263ra158–263ra158.

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstock, J., and Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America*, 108(38):16050–5.

Bravo, J. A., Julio-Pieper, M., Forsythe, P., Kunze, W., Dinan, T. G., Bienenstock, J., and Cryan, J. F. (2012). Communication between gastrointestinal bacteria and the nervous system. *Current Opinion in Pharmacology*, 12(6):667–672.

Browne, K. D., Chen, X.-H., Meaney, D. F., and Smith, D. H. (2011). Mild traumatic brain injury and diffuse axonal injury in Swine. *Journal of neurotrauma*, 28(9):1747–1755.

Bruzzese, E., Volpicelli, M., Squeglia, V., Bruzzese, D., Salvini, F., Bisceglia, M., Lionetti, P., Cinquetti, M., Iacono, G., Amarri, S., and Guarino, A. (2009). A formula containing galacto- and fructo-oligosaccharides prevents intestinal and extra-intestinal infections: An observational study. *Clinical Nutrition*, 28(2):156–161.

Buschhüter, D., Smitka, M., Puschmann, S., Gerber, J., Witt, M., Abolmaali, N., and Hummel, T. (2008). Correlation between olfactory bulb volume and olfactory function. *NeuroImage*, 42(2):498–502.

Bushnell, P. and Strupp, B. (2009). Assessing Attention in Rodents. In Buccafusco, J., editor, *Methods of Behavior Analysis in Neuroscience*, chapter 7. CRC Press, Boca Raton, 2 edition.

Campbell, B. and Coulter, X. (1976). Neural and psychological processes underlying the development of learning and memory. In Tighe, T. and Leaton, R., editors, *Habituation*, pages 129–157. NJ: Lawrence Erlbaum Associates, Inc., Hillsdale.

Cavallaro, S., Manickam, P., Dufour, F., and Alkon, D. L. (2002). Memory-specific temporal profiles of gene expression in the hippocampus. *National Institutes of Health*.

Chen, D., Yang, X., Yang, J., Lai, G., Yong, T., Tang, X., Shuai, O., Zhou, G., Xie, Y., and Wu, Q. (2017). Prebiotic Effect of Fructooligosaccharides from Morinda officinalis on Alzheimer's Disease in Rodent Models by Targeting the Microbiota-Gut-Brain Axis. *Frontiers in Aging Neuroscience*, 9:403.

Chen, Y., Zheng, Z., Zhu, X., Shi, Y., Tian, D., Zhao, F., Liu, N., Hüppi, P. S., Troy, F. A., and Wang, B. (2015). Lactoferrin Promotes Early Neurodevelopment and Cognition in Postnatal Piglets by Upregulating the BDNF Signaling Pathway and Polysialylation. *Molecular Neurobiology*, 52(1):256–269.

Chichlowski, M., Morairty, S., and Berg, B. M. (2017). Early life diet alters sleep architecture following an acute stress: The potential role of milk oligosaccharides. *FASEB Journal*, 31:636.22.

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science (New York, N.Y.)*, 325(5942):834–40.

Clark, R. E., West, a. N., Zola, S. M., and Squire, L. R. (2001). Rats with lesions of the hippocampus are impaired on the delayed nonmatching-to-sample task. *Hippocampus*, 11(2):176–86.

Clarke, G., Grenham, S., Scully, P., Fitzgerald, P., Moloney, R., Shanahan, F., Dinan, T., and Cryan, J. (2013). The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Molecular Psychiatry*, 18(10):666–673.

Clarke, G., O'Mahony, S. M., Dinan, T. G., and Cryan, J. F. (2014). Priming for health: gut microbiota acquired in early life regulates physiology, brain and behaviour. *Acta paediatrica (Oslo, Norway : 1992)*, 103(8):812–819.

Clevenger, M. A., Turnbull, D., Inoue, H., Enomoto, M., Allen, J. A., Henderson, L. M., and Jones3, E. (1988). Toxicological Evaluation of Neosugar : Genotoxicity, Carcinogenicity, and Chronic Toxicity. *JOURNAL OF THE AMERICAN COLLEGE OF TOXICOLOGY*, 7(5).

Colombo, J. (1993). *Infant cognition: Predicting later intellectual functioning*. Sage Publications, Inc., Newbury Park.

Colombo, J. and Cheatham, C. L. (2006). The Emergence and Basis of Endogenous Attention in Infancy and Early Childhood. In *Advances in Child Development and Behavior*, pages 283–322.

Colombo, J. and Mitchell, D. (1990). Individual and developmental differences in infant visual attention: Fixation time and ifnormation processing. In Colombo, J. and Fagen, J., editors, *Individual differences in infancy: Reliability, stability, and prediction*, pages 193–227. Lawrence Erlbaum, Hillsdale.

Colombo, J., Mitchell, D., Coldren, J., and Freeseman, L. (1991). Individual differences in infant attention: Are short lookers faster processors or feature processors? *Child Development*, 62:1247–1257.

Colombo, J., Shaddy, D. J., Richman, W. A., Maikranz, J. M., and Blaga, O. M. (2004). Developmental course of habituation in infancy and preschool outcome. *Infancy*, 5(1):1–38.

Comstock, S. S., Li, M., Wang, M., Monaco, M. H., Kuhlenschmidt, T. B., Kuhlenschmidt, M. S., and Donovan, S. M. (2017). Dietary Human Milk Oligosaccharides but Not Prebiotic Oligosaccharides Increase Circulating Natural Killer Cell and Mesenteric Lymph Node Memory T Cell Populations in Noninfected and Rotavirus-Infected Neonatal Piglets. *The Journal of Nutrition*, 147(6):1041–1047.

Conrad, M. S., Dilger, R. N., and Johnson, R. W. (2012a). Brain growth of the domestic pig (Sus scrofa) from 2 to 24 weeks of age: a longitudinal MRI study. *Developmental neuroscience*, 34(4):291–8.

Conrad, M. S., Dilger, R. N., Nickolls, A., and Johnson, R. W. (2012b). Magnetic resonance imaging of the neonatal piglet brain. *Pediatric research*, 71(2):179–84.

Conrad, M. S., Sutton, B. P., Dilger, R. N., and Johnson, R. W. (2014). An in vivo three-dimensional magnetic resonance imaging-based averaged brain collection of the neonatal piglet (Sus scrofa). *PloS one*, 9(9):e107650.

Cooper, P., Bolton, K. D., Velaphi, S., De Groot, N., Emady-Azar, S., Pecquet, S., and Steenhout, P. (2016). Early Benefits of a Starter Formula Enriched in Prebiotics and Probiotics on the Gut Microbiota of Healthy Infants Born to HIV+ Mothers: A Randomized Double-Blind Controlled Trial. *Clinical Medicine Insights: Pediatrics*, 10:CMPed.S40134.

Costabile, A., Fava, F., Röytiö, H., Forssten, S. D., Olli, K., Klievink, J., Rowland, I. R., Ouwehand, A. C., Rastall, R. a., Gibson, G. R., and Walton, G. E. (2012). Impact of polydextrose on the faecal microbiota: a double-blind, crossover, placebo-controlled feeding study in healthy human subjects. *The British journal of nutrition*, 108(3):471–81.

Costalos, C., Kapiki, A., Apostolou, M., and Papathoma, E. (2008). The effect of a prebiotic supplemented formula on growth and stool microbiology of term infants. *Early Human Development*, 84(1):45–49.

Croney, C., Adams, K., Washington, C., and Stricklin, W. (2003). A note on visual, olfactory and spatial cue use in foraging behavior of pigs: Indirectly assessing cognitive abilities. *Applied Animal Behaviour Science*, 83(4):303–308.

Crumeyrolle-Arias, M., Jaglin, M., Bruneau, A., Vancassel, S., Cardona, A., Daugé, V., Naudon, L., and Rabot, S. (2014). Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. *Psychoneuroendocrinology*, 42:207–217.

Cummings, D. M., Knab, B. R., and Brunjes, P. C. (1997). Effects of unilateral olfactory deprivation in the developing opossum,Monodelphis domestica. *Journal of Neurobiology*, 33(4):429–438.

Cummings, J. and Macfarlane, G. (1991). The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, 70(6):443–459.

Dai, Z.-L., Wu, G., and Zhu, W.-Y. (2011). Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. *Frontiers in Bioscience*, 16:1768–1786.

den Besten, G., van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., and Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res*, 54(9):2325–2340.

Deoni, S., Dean, D., Joelson, S., O'Regan, J., and Schneider, N. (2018). Early nutrition influences developmental myelination and cognition in infants and young children. *NeuroImage*, 178:649–659.

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T. G., and Cryan, J. F. (2014). Microbiota is essential for social development in the mouse. *Molecular psychiatry*, 19(2):146–8.

Desbonnet, L., Garrett, L., Clarke, G., Kiely, B., Cryan, J. F., and Dinan, T. G. (2010). Effects of the probiotic Bifidobacterium infantis in the maternal separation model of depression. *Neuroscience*, 170(4):1179–1188.

Dickerson, J. and Dobbing, J. (1966). Prenatal and postnatal growth and development of the central nervous system of the pig. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 166(1005):384–395.

Dilger, R. N. and Johnson, R. W. (2010). Behavioral assessment of cognitive function using a translational neonatal piglet model. *Brain, behavior, and immunity*, 24(7):1156–65.

Dinan, T. G. and Cryan, J. F. (2012). Regulation of the stress response by the gut microbiota: Implications for psychoneuroendocrinology. *Psychoneuroendocrinology*, 37(9):1369–1378.

Dionex (2009). Determination of catecholamines in human plasma by liquid chromatography with electrochemical detection. Technical report, Thermo Scientific.

do Carmo, M. M. R., Walker, J. C. L., Novello, D., Caselato, V. M., Sgarbieri, V. C., Ouwehand, A. C., Andreollo, N. A., Hiane, P. A., and dos Santos, E. F. (2016). Polydextrose: physiological function, and effects on health. *Nutrients*, 8(9):1–13.

Dobbing, J. and Sands, J. (1979). Comparative aspects of the brain growth spurt. *Early Human Development*, 3(1):79–83.

Drai, D., Kafkafi, N., Benjamini, Y., Elmer, G., and Golani, I. (2001). Rats and mice share common ethologically relevant parameters of exploratory behavior. *Behavioural Brain Research*, 125(1-2):133–140.

Duberstein, K. J., Platt, S. R., Holmes, S. P., Dove, C. R., Howerth, E. W., Kent, M., Stice, S. L., Hill, W. D., Hess, D. C., and West, F. D. (2014). Gait analysis in a pre- and post-ischemic stroke biomedical pig model. *Physiology & behavior*, 125:8–16.

Edwards, C. A. and Eastwood, M. A. (1995). Caecal and faecal short-chain fatty acids and stool output in rats fed on diets containing non-starch polysaccharides. *British Journal of Nutrition*, 73(5):773–781.

Elmore, M. R. P., Dilger, R. N., and Johnson, R. W. (2013). Place and direction learning in a spatial T-maze task by neonatal piglets. *Animal Cognition*, 15(4):667–676.

Ennaceur, A. and Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. *Behavioral Brain Research*, 31:47–59.

Ennaceur, A., Neave, N., and Aggleton, J. P. (1996). Neurotoxic lesions of the perirhinal cortex do not mimic the behavioural effects of fornix transection in the rat. *Behavioural Brain Research*, 80(1-2):9–25.

Erny, D., Hrabě de Angelis, A. L., Jaitin, D., Wieghofer, P., Staszewski, O., David, E., Keren-Shaul, H., Mahlakoiv, T., Jakobshagen, K., Buch, T., Schwierzeck, V., Utermöhlen, O., Chun, E., Garrett, W. S., McCoy, K. D., Diefenbach, A., Staeheli, P., Stecher, B., Amit, I., and Prinz, M. (2015). Host microbiota constantly control maturation and function of microglia in the CNS. *Nature Neuroscience*, 18(7):965–977.

Fagan, J. I. (1973). Infants ' Delayed Recognition. *Journal of Experimental Child Psychology*, 424450:424–450.

Fagioli, S., Castellano, C., Oliverio, A., and Toffano, G. (1990). Effect of chronic GM1 ganglioside administration on passive avoidance retention in mice. *Neuroscience Letters*, 109(1-2):212–216.

Fagioli, S., Rossi-Arnaud, C., and Castellano, C. (1992). Dose-dependent effect of GM1 ganglioside during development on inhibitory avoidance behaviour in mice: influence of the period of administration. *Psychopharmacology*, 109(4):457–60.

Fantz, R. L. (1956). A method for studying early visual development. *Perceptual and Motor Skills*, 6:13–15.

Fava, F., Mäkivuokko, H., Siljander-Rasi, H., Putaala, H., Tiihonen, K., Stowell, J., Tuohy, K., Gibson, G., and Rautonen, N. (2007). Effect of polydextrose on intestinal microbes and immune functions in pigs. *Br. J. Nutr.*, 98(2007):123–133.

Fischer, A., Sananbenesi, F., Wang, X., Dobbin, M., and Tsai, L.-H. (2007). Recovery of learning and memory is associated with chromatin remodelling. *Nature*, 447(7141):178–182.

Fleming, S. A., Chichlowski, M., Berg, B. M., Donovan, S. M., and Dilger, R. N. (2018). Dietary Sialyllactose Does Not Influence Measures of Recognition Memory or Diurnal Activity in the Young Pig. *Nutrients*, 10(4):395.

Fleming, S. A. and Dilger, R. N. (2017). Young pigs exhibit differential exploratory behavior during novelty preference tasks in response to age, sex, and delay. *Behavioural Brain Research*, 321:50–60.

Fleming, S. A., Monaikul, S., Patsavas, A. J., Waworuntu, R. V., Berg, B. M., and Dilger, R. N. (2017). Dietary polydextrose and galactooligosaccharide increase exploratory behavior, improve recognition memory, and alter neurochemistry in the young pig. *Nutritional Neuroscience*, 0(0):1–14.

Fong, T. G., Neff, N. H., and Hadjiconstantinou, M. (1997). GM1 ganglioside improves spatial learning and memory of aged rats. *Behavioural brain research*, 85:203–211.

Forsythe, P., Bienenstock, J., and Kunze, W. A. (2014). Vagal Pathways for Microbiome-Brain-Gut Axis Communication. In *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease*, pages 115–133.

Freitas dos Santos, E., Hitomi Tsuboi, K., Araújo, M. R., C. Ouwehand, A., Adami Andreollo, N., and Kenji Miyasaka, C. (2009). Dietary polydextrose increases calcium absortion in normal rats. *ABCD. Surgery Brazilian Archives of Digestive Surgery*, 22(4):201–205.

Friess, S. H., Ichord, R. N., Owens, K., Ralston, J., Rizol, R., Overall, K. L., Smith, C., Helfaer, M. a., and Margulies, S. S. (2007). Neurobehavioral functional deficits following closed head injury in the neonatal pig. *Experimental neurology*, 204(1):234–43.

Frost, G., Sleeth, M. L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., Anastasovska, J., Ghourab, S., Hankir, M., Zhang, S., Carling, D., Swann, J. R., Gibson, G., Viardot, A., Morrison, D., Louise Thomas, E., and Bell, J. D. (2014). The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nature Communications*, 5(1):3611.

García-Villalba, R., Giménez-Bastida, J. A., García-Conesa, M. T., Tomás-Barberán, F. A., Carlos Espín, J., and Larrosa, M. (2012). Alternative method for gas chromatography-mass spectrometry analysis of short-chain fatty acids in faecal samples. *Journal of Separation Science*, 35(15):1906–1913.

Gershon, M. D. and Tack, J. (2007). The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology*, 132(1):397–414.

Getty, C. M. and Dilger, R. N. (2015). Moderate perinatal choline deficiency elicits altered physiology and metabolomic profiles in the piglet. *PLoS ONE*, 10(7):1–11.

Gibson, G. R., Scott, K. P., Rastall, R. A., Tuohy, K. M., Hotchkiss, A., Dubert-Ferrandon, A., Gareau, M., Murphy, E. F., Saulnier, D., Loh, G., Macfarlane, S., Delzenne, N., Ringel, Y., Kozianowski, G., Dickmann, R., Lenoir-Wijnkook, I., Walker, C., and Buddington, R. (2010). Dietary prebiotics: current status and new definition. *Food Science and Technology Bulletin: Functional Foods*, 7(1):1–19.

Gieling, E. T., Nordquist, R. E., and van der Staay, F. J. (2011a). Assessing learning and memory in pigs. *Animal Cognition*, 14(2):151–173.

Gieling, E. T., Schuurman, T., Nordquist, R. E., and van der Staay, F. J. (2011b). The pig as a model animal for studying cognition and neurobehavioral disorders. *Molecular and Functional Models in Neuropsychiatry*.

Gifford, A. K., Cloutier, S., and Newberry, R. C. (2007). Objects as enrichment: Effects of object exposure time and delay interval on object recognition memory of the domestic pig. *Applied Animal Behaviour Science*, 107(3-4):206–217.

Girard, L.-C., Doyle, O., and Tremblay, R. E. (2017). Breastfeeding, Cognitive and Noncognitive Development in Early Childhood: A Population Study. *Pediatrics*, 139(4):e20161848.

Glaser, D., Wanner, M., Tinti, J. M., and Nofre, C. (2000). Gustatory responses of pigs to various natural and artificial compounds known to be sweet in man. *Food Chemistry*, 68:375–385.

Glasier, M. M., Janis, L. S., Goncalves, M. I., and Stein, D. G. (1999). GM1 produces attenuation of short-term memory deficits in Hebb-Williams maze performance after unilateral entorhinal cortex lesions. *Physiology and Behavior*, 66(3):441–446.

Goehler, L. E., Gaykema, R. P. A., Opitz, N., Reddaway, R., Badr, N., and Lyte, M. (2005). Activation in vagal afferents and central autonomic pathways: Early responses to intestinal infection with Campylobacter jejuni. *Brain, Behavior, and Immunity*, 19(4):334–344.

Goehring, K. C., Marriage, B. J., Oliver, J. S., Wilder, J. A., Barrett, E. G., and Buck, R. H. (2016). Similar to Those Who Are Breastfed , Infants Fed a Formula Containing 2 # -Fucosyllactose Have Lower Inflammatory Cytokines in a Randomized Controlled Trial. *The Journal of nutrition*, 146(12):2559–66.

Gopal, P. K. and Gill, H. S. (2000). Oligosaccharides and glycoconjugates in bovine milk and colostrum. *British Journal of Nutrition*, 84(S1).

Gordon, S. L. and Cousin, M. A. (2014). The Sybtraps: control of synaptobrevin traffic by synaptophysin, α-synuclein and AP-180. *Traffic*, 15(3):245–254.

Gosso, F. M., de Geus, E. J., Polderman, T. J., Boomsma, D. I., Posthuma, D., and Heutink, P. (2007). Exploring the functional role of the CHRM2 gene in human cognition: results from a dense genotyping and brain expression study. *BMC Medical Genetics*, 8(1):66.

Grimaldi, R., Cela, D., Swann, J. R., Vulevic, J., Gibson, G. R., Tzortzis, G., and Costabile, A. (2017). In vitro fermentation of B-GOS: impact on faecal bacterial populations and metabolic activity in autistic and non-autistic children. *FEMS Microbiology Ecology*, 93(2):1–10.

Gronier, B., Savignac, H. M., Miceli, M. D., Idriss, S. M., Tzortzis, G., Anthony, D., and Burnet, P. W. J. (2018). Increased cortical neuronal responses to NMDA and improved attentional set-shifting performance in rats following prebiotic (B-GOS s) ingestion.

Gurnida, D. A., Rowan, A. M., Idjradinata, P., Muchtadi, D., and Sekarwana, N. (2012). Association of complex lipids containing gangliosides with cognitive development of 6-month-old infants. *Early Human Development*, 88(8):595–601.

Gustavsson, M., Hodgkinson, S. C., Fong, B., Norris, C., Guan, J., Krageloh, C. U., Breier, B. H., Davison, M., McJarrow, P., and Vickers, M. H. (2010). Maternal supplementation with a complex milk lipid mixture during pregnancy and lactation alters neonatal brain lipid composition but lacks effect on cognitive function in rats. *Nutrition Research*, 30(4):279–289.

Haettig, J., Stefanko, D. P., Multani, M. L., Figueroa, D. X., McQuown, S. C., and Wood, M. A. (2011). HDAC inhibition modulates hippocampus-dependent long-term memory for object location in a CBP-dependent manner. *Learning & memory (Cold Spring Harbor, N.Y.)*, 18(2):71–9.

Hammell, D., Kratzer, D., and Bramble, W. (1975). Avoidance and Maze Learning in Pigs. *Journal of animal science*, 40(3):573–9.

Hammond, R. S., Tull, L. E., and Stackman, R. W. (2004). On the delay-dependent involvement of the hippocampus in object recognition memory. *Neurobiology of learning and memory*, 82(1):26–34.

Hanstock, T. L., Clayton, E. H., Li, K. M., and Mallet, P. E. (2004). Anxiety and aggression associated with the fermentation of carbohydrates in the hindgut of rats. *Physiology and Behavior*, 82(2-3):357–368.

Hanstock, T. L., Mallet, P. E., and Clayton, E. H. (2010). Increased plasma d-lactic acid associated with impaired memory in rats. *Physiology and Behavior*, 101(5):653–659.

Hartshorn, K., Rovee-Collier, C., Gerhardstein, P., Bhatt, R. S., Wondoloski, T. L., Klein, P., Gilch, J., Wurtzel, N., and Campos-de Carvalho, M. (1998). The ontogeny of long-term memory over the first year-and-a-half of life. *Developmental Psychobiology*, 32(2):69–89.

Hartshorn, K., Rovee-collier, C., and Rove-Collier, C. (1997). Infant learning and long-term memory at 6 months: A confirming analysis. *Developmental Psychobiology*, 30:71–85.

Hayne, H. (2007). Infant memory development. In Oakes, L. and Bauer, editors, *Short- and long-term memory in infnacy and early childhood*, pages 209–239. New York: Oxford University Press.

Heaney, C. F. and Kinney, J. W. (2016). Role of GABABreceptors in learning and memory and neurological disorders. *Neuroscience and Biobehavioral Reviews*, 63:1–28.

Heffner, H. and Heffner, R. (1992). Auditory perception. In Phillips, C. and Piggins, D., editors, *Farm Animals and the Environment*, pages 159–184. C.A.B. International, Wallingford.

Heffner, R. and Heffner, H. (1990). Hearing in domestic pigs (Sus scrofa) and goats (Capra hircus). *Hearing Research*, 48:231–240.

Heijtz, R. D., Wang, S., Anuar, F., Qian, Y., Bj rkholm, B., Samuelsson, A., Hibberd, M. L., Forssberg, H., and Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences*, 108(7):3047–3052.

Held, S., Cooper, J. J., and Mendl, M. T. (2009). *The welfare of pigs*, volume 7 of *Animal Welfare*. Springer Netherlands, Dordrecht.

Held, S., Mendl, M., Devereux, C., and Byrne, R. (2000). Social tactics of pigs in a competitive foraging task: the 'informed forager' paradigm. *Animal behaviour*, 59(3):569–576.

Held, S., Mendl, M., Laughlin, K., and Byrne, R. W. (2002). Cognition studies with pigs : Livestock cognition and its implication for production. *Journal of Animal Science*, 80:e10–17.

Hemsworth, P., Price, E., and Borgwardt, R. (1996). Behavioral responses of domestic pigs and cattle to humans and novel stimuli. *Applied Animal Behavior Science*, 50:43–56.

Hengst, C., Ptok, S., Roessler, A., Fechner, A., and Jahreis, G. (2009). Effects of polydextrose supplementation on different faecal parameters in healthy volunteers. *International journal of food sciences and nutrition*, 60 Suppl 5(September):96–105.

Hernot, D. C., Boileau, T. W., Bauer, L. L., Middelbos, I. S., Murphy, M. R., Swanson, K. S., and Fahey, G. C. (2009). In Vitro Fermentation Profiles, Gas Production Rates, and Microbiota Modulation as Affected by Certain Fructans, Galactooligosaccharides, and Polydextrose. *Journal of Agricultural and Food Chemistry*, 57(4):1354–1361.

Hoffman, D. R., Boettcher, J. A., and Diersen-Schade, D. A. (2009). Toward optimizing vision and cognition in term infants by dietary docosahexaenoic and arachidonic acid supplementation: A review of randomized controlled trials. *Prostaglandins Leukotrienes and Essential Fatty Acids*, 81(2-3):151–158.

Horta, B. L., Loret De Mola, C., and Victora, C. G. (2015). Breastfeeding and intelligence: A systematic review and meta-analysis. *Acta Paediatrica, International Journal of Paediatrics*, 104:14–19.

Hummel, T., Smitka, M., Puschmann, S., Gerber, J. C., Schaal, B., and Buschhüter, D. (2011). Correlation between olfactory bulb volume and olfactory function in children and adolescents. *Experimental Brain Research*, 214(2):285–291.

Hutson, G., Ambrose, T., Barnett, J., and Tilbrook, A. (2000). Development of a behavioural test of sensory responsiveness in the growing pig. *Applied Animal Behaviour Science*, 66(3):187–202.

Intlekofer, K. A., Berchtold, N. C., Malvaez, M., Carlos, A. J., McQuown, S. C., Cunningham, M. J., Wood, M. A., and Cotman, C. W. (2013). Exercise and Sodium Butyrate Transform a Subthreshold Learning Event into Long-Term Memory via a Brain-Derived Neurotrophic factor-Dependent Mechanism. *Neuropsychopharmacology*, 38(10):2027–2034.

Jacobi, S. K., Yatsunenko, T., Li, D., Dasgupta, S., Yu, R. K., Berg, B. M., Chichlowski, M., and Odle, J. (2016). Dietary Isomers of Sialyllactose Increase Ganglioside Sialic Acid Concentrations in the Corpus Callosum and Cerebellum and Modulate the Colonic Microbiota of Formula-Fed Piglets. *The Journal of nutrition*, 146(2):200–8.

Janik, R., Thomason, L. A., Stanisz, A. M., Forsythe, P., Bienenstock, J., and Stanisz, G. J. (2016). Magnetic resonance spectroscopy reveals oral Lactobacillus promotion of increases in brain GABA, N-acetyl aspartate and glutamate. *NeuroImage*, 125:988–995.

Jia, S., Lu, Z., Gao, Z., An, J., Wu, X., Li, X., Dai, X., Zheng, Q., and Sun, Y. (2016). Chitosan oligosaccharides alleviate cognitive deficits in an amyloid-β1–42-induced rat model of Alzheimer's disease. *International Journal of Biological Macromolecules*, 83:416–425.

Jones, J., Carmichael, N., Wathes, C., White, R., and Jones, R. (2000). The effects of acute simultaneous exposure to ammonia on the detection of buried odourized food by pigs. *Applied Animal Behaviour Science*, 65(4):305–319.

Jork, R., Grecksch, G., and Matthies, H. (1986). Impairment of glycoprotein fucosylation in rat hippocampus and the consequences on memory formation. *Pharmacology, Biochemistry and Behavior*, 25(6):1137–1144.

Kao, A. C.-C., Chan, K. W., Anthony, D. C., Lennox, B. R., and Burnet, P. W. (2019). Prebiotic reduction of brain histone deacetylase (HDAC) activity and olanzapine-mediated weight gain in rats, are acetate independent. *Neuropharmacology*, 150:184–191.

Kao, A. C. C., Harty, S., and Burnet, P. W. J. (2016). *The Influence of Prebiotics on Neurobiology and Behavior*, volume 131. Elsevier Inc., 1 edition.

Kapiki, A., Costalos, C., Oikonomidou, C., Triantafyllidou, A., Loukatou, E., and Pertrohilou, V. (2007). The effect of a fructo-oligosaccharide supplemented formula on gut flora of preterm infants. *Early Human Development*, 83(5):335–339.

Kennedy, J. and Baldwin, B. (1972). Taste preference in pigs for nutritive and non-nutritive sweet solutions. *Animal Behaviour*, 20:706–718.

Khan, N. A., Raine, L. B., Drollette, E. S., Scudder, M. R., Kramer, A. F., and Hillman, C. H. (2015). Dietary Fiber Is Positively Associated with Cognitive Control among Prepubertal. *The Journal of Nutrition: Ingestive Behavior and Neurosciences*, 145(1):143–149.

Kim, H. J., Leeds, P., and Chuang, D.-M. (2009). The HDAC inhibitor, sodium butyrate, stimulates neurogenesis in the ischemic brain. *Journal of Neurochemistry*, 110(4):1226–1240.

Kirmiz, N., Robinson, R. C., Shah, I. M., Barile, D., and Mills, D. A. (2018). Milk Glycans and Their Interaction with the Infant-Gut Microbiota. *Annual Review of Food Science and Technology*, 9(1):429–450.

Knickmeyer, R. C., Gouttard, S., Kang, C., Evans, D., Wilber, K., Smith, J. K., Hamer, R. M., Lin, W., Gerig, G., and Gilmore, J. H. (2008). A Structural MRI study of human brain development from birth to 2 years. *Journal of Neuroscience*, 28(47):12176–12182.

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Bäckhed, F. (2016). From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell*, 165(6):1332–1345.

Konings, E., Schoffelen, P. F., Stegen, J., and Blaak, E. E. (2014). Effect of polydextrose and soluble maize fibre on energy metabolism, metabolic profile and appetite control in overweight men and women. *The British journal of nutrition*, 111(1):111–21.

Konsman, J. P., Luheshi, G. N., Bluthé, R.-M., and Dantzer, R. (2000). The vagus nerve mediates behavioural depression, but not fever, in response to peripheral immune signals; a functional anatomical analysis. *European Journal of Neuroscience*, 12(12):4434–4446.

Kornum, B. R. and Knudsen, G. M. (2011). Cognitive testing of pigs (Sus scrofa) in translational biobehavioral research. *Neuroscience and Biobehavioral Reviews*, 35(3):437–451.

Kornum, B. R., Thygesen, K. S., Nielsen, T. R., Knudsen, G. M., and Lind, N. M. (2007). The effect of the inter-phase delay interval in the spontaneous object recognition test for pigs. *Behavioural brain research*, 181(2):210–7.

Korol, D. and Brunjes, P. (1992). Unilateral naris closure and vascular development in the rat olfactory bulb. *Neuroscience*, 46(3):631–641.

Krahl, S. E., Senanayake, S. S., Pekary, A. E., and Sattin, A. (2004). Vagus nerve stimulation (VNS) is effective in a rat model of antidepressant action. *Journal of psychiatric research*, 38(3):237–40.

Kratzer, D. D. (1969). Effects of age on avoidance learning in pigs. *Journal of Animal Science*, 28:175–179.

Kressel, M. and Radespiel-Tröger, M. (1999). Anterograde tracing and immunohistochemical characterization of potentially mechanosensitive vagal afferents in the esophagus. *The Journal of Comparative Neurology*, 412(1):161–172.

Kristensen, H. H., Jones, R. B., Schofield, C. P., White, R. P., and Wathes, C. M. (2001). The use of olfactory and other cues for social recognition by juvenile pigs. *Applied animal behaviour science*, 72(4):321–333.

Krug, M., Wagner, M., Staak, S., and Smalla, K.-H. (1994). Fucose and fucose-containing sugar epitopes enhance hippocampal long-term potentiation in the freely moving rat. *BRAIN RESEARCH ELSEVIER Brain Research*, 643:130–135.

Kyriazakis, I., Emmans, G. C., and Whittemore, C. T. (1990). Diet selection in pigs- choices made by growing pigs given foods of different concentrations. *Animal Production*, 51(01):189–199.

Lal, S., Kirkup, a. J., Brunsden, a. M., Thompson, D. G., and Grundy, D. (2001). Vagal afferent responses to fatty acids of different chain length in the rat. *American journal of physiology. Gastrointestinal and liver physiology*, 281(4):G907–G915.

Larsen, M. O. and Rolin, B. (2004). Use of the Göttingen minipig as a model of diabetes, with special focus on type 1 diabetes research. *ILAR journal / National Research Council, Institute of Laboratory Animal Resources*, 45(3):303–313.

Lattal, K. M., Barrett, R. M., and Wood, M. A. (2007). Systemic or intrahippocampal delivery of histone deacetylase inhibitors facilitates fear extinction. *Behavioral Neuroscience*, 121(5):1125–1131.

Laughlin, K. and Mendl, M. (2000). Pigs shift too: foraging strategies and spatial memory in the domestic pig. *Animal behaviour*, 60(3):403–410.

Lawson, K. and Ruff, H. (2004a). Early attention and negative emotionality predict later cognitive and behavioral function. *International Journal of Behavioral Development*, 28:157–165.

Lawson, K. and Ruff, H. (2004b). Early focused attention predicts outcome for children born prematurely. *Journal of Developmental and Behavioral Pediatrics*, 25:399–406.

Legette, L. L., Lee, W., Martin, B. R., Story, J. A., Campbell, J. K., and Weaver, C. M. (2012). Prebiotics Enhance Magnesium Absorption and Inulin-based Fibers Exert Chronic Effects on Calcium Utilization in a Postmenopausal Rodent Model. *Journal of Food Science*, 77(4):88–94.

Lehmann, H. (1998). The minipig in general toxicology. *Scandinavian Journal of Laboratory Animal Science*, 25:59–62.

Levay, P. F. and Viljoen, M. (1995). Lactoferrin: A general review. *Haematologica*, 80:252–267.

Levrat, M.-a., Rãšmã, C., and Demignã, C. (1991). Carbohydrates and Fibers High Propionic Acid Fermentations and Mineral Accumulation in the Cecum of Rats Adapted to Different Levels of Inulin. Technical report.

Li, M., Bauer, L. L., Chen, X., Wang, M., Kuhlenschmidt, T. B., Kuhlenschmidt, M. S., Fahey, G. C., and Donovan, S. M. (2012). Microbial Composition and In Vitro Fermentation Patterns of Human Milk Oligosaccharides and Prebiotics Differ between Formula-Fed and Sow-Reared Piglets. *Journal of Nutrition*, 142(4):681–689.

Li, Y. and Li, Y. (2007). Sensory Signal Transduction in the Vagal Primary Afferent Neurons. *Current Medicinal Chemistry*, 14(24):2554–2563.

Lind, N. M., Moustgaard, A., Jelsing, J., Vajta, G., Cumming, P., and Hansen, A. K. (2007). The use of pigs in neuroscience: modeling brain disorders. *Neuroscience and biobehavioral reviews*, 31(5):728–51.

Liu, H., Radlowski, E. E. C., Conrad, M. M. S., Li, Y., Dilger, R. N., and Johnson, R. W. (2014). Early supplementation of phospholipids and gangliosides affects brain and cognitive development in neonatal piglets. *The Journal of Nutrition*, 144(12):1–7.

Liu, J., Sun, J., Wang, F., Yu, X., Ling, Z., Li, H., Zhang, H., Jin, J., Chen, W., Pang, M., Yu, J., He, Y., and Xu, J. (2015). Neuroprotective Effects of Clostridium butyricum against Vascular Dementia in Mice via Metabolic Butyrate. *BioMed research international*, 2015:412946.

Lönnerdal, B. (2014). Infant formula and infant nutrition: Bioactive proteins of human milk and implications for composition of infant formulas. *American Journal of Clinical Nutrition*, 99(3).

Luheshi, G. N., Bluthé, R.-M., Rushforth, D., Mulcahy, N., Konsman, J.-P., Goldbach, M., and Dantzer, R. (2000). Vagotomy attenuates the behavioural but not the pyrogenic effects of interleukin-1 in rats. *Autonomic Neuroscience*, 85(1-3):127–132.

Luo, J., Wang, T., Liang, S., Hu, X., Li, W., and Jin, F. (2014). Ingestion of Lactobacillus strain reduces anxiety and improves cognitive function in the hyperammonemia rat. *Science China Life Sciences*, 57(3):327–335.

Lyte, M. (2013). Microbial Endocrinology in the Microbiome-Gut-Brain Axis: How Bacterial Production and Utilization of Neurochemicals Influence Behavior. *PLoS Pathogens*, 9(11).

Lyte, M., Varcoe, J. J., and Bailey, M. T. (1998). Anxiogenic effect of subclinical bacterial infection in mice in the absence of overt immune activation. *Physiology & Behavior*, 65(1):63–68.

Macfabe, D. F. (2012). Short-chain fatty acid fermentation products of the gut microbiome: implications in autism spectrum disorders. *Microbial ecology in health and disease*, 23:1–24.

Macfarlane, G. T. and Macfarlane, S. (2011). Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics. *Journal of clinical gastroenterology*, 45 Suppl:S120–7.

Macfarlane, G. T., Steed, H., and Macfarlane, S. (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology*, 104(2):305–344.

Macfarlane, S. and Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. *Proceedings of the Nutrition Society*, 62(01):67–72.

Mäkeläinen, H., Ottman, N., Forssten, S., Saarinen, M., Rautonen, N., and Ouwehand, A. C. (2010). Synbiotic effects of galacto-oligosaccharide, polydextrose and Bifidobacterium lactis Bi-07 in vitro. *International Journal of Probiotics and Prebiotics*, 5(4):203–210.

Mäkeläinen, H. S., Mäkivuokko, H. A., Salminen, S. J., Rautonen, N. E., and Ouwehand, A. C. (2007). The effects of polydextrose and xylitol on microbial community and activity in a 4-stage colon simulator. *Journal of Food Science*, 72(5):153–159.

Marriage, B. J., Buck, R. H., Goehring, K. C., Oliver, J. S., and Williams, J. A. (2015). Infants Fed a Lower Calorie Formula with 2 FL Show Growth and 2 FL Uptake Like Breast-Fed Infants. *Journal of Pediatric Gastroenterology and Nutrition*, 61(6):649–658.

Martin, C., Ling, P.-R., and Blackburn, G. (2016). Review of infant feeding: key features of breast milk and infant formula. *Nutrients*, 8(5):279.

Martin, C. R., Osadchiy, V., Kalani, A., and Mayer, E. A. (2018). The Brain-Gut-Microbiome Axis. *Cellular and molecular gastroenterology and hepatology*, 6(2):133–148.

Martín-Sosa, S., Martín, M.-J., García-Pardo, L.-A., and Hueso, P. (2003). Sialyloligosaccharides in Human and Bovine Milk and in Infant Formulas: Variations with the Progression of Lactation. *Journal of Dairy Science*, 86(1):52–59.

Martín-Sosa, S., Martín, M.-J., García-Pardo, L. a., and Hueso, P. (2004). Distribution of sialic acids in the milk of spanish mothers of full term infants during lactation. *Journal of pediatric gastroenterology and nutrition*, 39(10):499–503.

Matsumoto, M., Kibe, R., Ooga, T., Aiba, Y., Kurihara, S., Sawaki, E., Koga, Y., and Benno, Y. (2012). Impact of intestinal microbiota on intestinal luminal metabolome. *Scientific reports*, 2:233.

Matthies, H., Staak, S., and Krug, M. (1996). Fucose and fucosyllactose enhance in-vitro hippocampal long-term potentiation. *Brain Research*, 725(2):276–280.

McCall, R. and Carriger, M. (1993). A meta-analysis of infant habituation and recognition memory performance as predictors of later IQ. *Child Development*, 64:57–79.

McGlone, J. (1990). Olfactory signals that modulate pig aggressive and submissive behavior. In Zayan, R. and Dantzer, R., editors, *Social Stress in Domestic Animals*, pages 86–109. Kluwer Academic Press, Dordrecht.

Mclaughlin, C. L., Baile, C. A., Buckholtz, L. L., and Freeman, S. K. (1983). Preferred flavors and performance of weanling pigs. *Journal of animal science*, pages 1287–1293.

Meese, G., Conner, D., and Baldwin, B. (1975). Ability of the pig to distinguish between conspecific urine samples using olfaction. *Physiology & behavior*, 15(1):121–5.

Meli, F., Puccio, G., Cajozzo, C., Ricottone, G. L., Pecquet, S., Sprenger, N., and Steenhout, P. (2014). Growth and safety evaluation of infant formulae containing oligosaccharides derived from bovine milk: a randomized, double-blind, noninferiority trial. *BMC pediatrics*, 14:306.

Mendez, M., Arias, N., Uceda, S., and Arias, J. L. (2015). c-Fos expression correlates with performance on novel object and novel place recognition tests. *Brain research bulletin*, 117:16–23.

Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A., Bisson, J.-F., Rougeot, C., Pichelin, M., Cazaubiel, M., and Cazaubiel, J.-M. (2011a). Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *The British journal of nutrition*, 105(5):755–764.

Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A., Bisson, J.-F., Rougeot, C., Pichelin, M., Cazaubiel, M., and Cazaubiel, J.-M. (2011b). Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *The British journal of nutrition*, 105(5):755–764.

Mika, A., Day, H. E., Martinez, A., Rumian, N. L., Greenwood, B. N., Chichlowski, M., Berg, B. M., and Fleshner, M. (2017). Early life diets with prebiotics and bioactive milk fractions attenuate the impact of stress on learned helplessness behaviours and alter gene expression within neural circuits important for stress resistance. *European Journal of Neuroscience*, 45(3):342–357.

Millet, S., Van Oeckel, M. J., Aluwé, M., Delezie, E., De Brabander, D. L., and Aluwé, A. (2010). Prediction of In Vivo Short-Chain Fatty Acid Production in Hindgut Fermenting Mammals: Problems and Pitfalls. *Critical Reviews in Food Science and Nutrition*, 50(7):605–619.

Minamiyama, M., Katsuno, M., Adachi, H., Waza, M., Sang, C., Kobayashi, Y., Tanaka, F., Doyu, M., Inukai, A., and Sobue, G. (2004). Sodium butyrate ameliorates phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Human molecular genetics*, 13(11):1183–92.

Moore, A. and Marcuse, F. (1945). Salivary, cardiac and motor indices of conditioning in two sows. *Journal of Comparative Psychology*, 38:1–16.

Morgan, B. L. and Winick, M. (1980). Effects of administration of N-acetylneuraminic acid (NANA) on brain NANA content and behavior. *The Journal of nutrition*, 110(3):416–24.

Morrow, A. L., Ruiz-Palacios, G. M., Jiang, X., and Newburg, D. S. (2005). Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. *The Journal of nutrition*, 135(5):1304–1307.

Moustgaard, A., Arnfred, S. M., Lind, N. M., Hemmingsen, R., and Hansen, A. K. (2005). Acquisition of visually guided conditional associative tasks in Göttingen minipigs. *Behavioural processes*, 68(1):97–102.

Moustgaard, A., Lind, N. M., Hemmingsen, R., and Hansen, A. K. (2002). Spontaneous object recognition in the Göttingen minipig. *Neural plasticity*, 9(4):255–9.

Mudd, A., Fil, J., Knight, L., Lam, F., Liang, Z.-P., and Dilger, R. (2018a). Early-Life Iron Deficiency Reduces Brain Iron Content and Alters Brain Tissue Composition Despite Iron Repletion: A Neuroimaging Assessment. *Nutrients*, 10(2):135.

Mudd, A. T., Alexander, L. S., Berding, K., Waworuntu, R. V., Berg, B. M., Donovan, S. M., and Dilger, R. N. (2016a). Dietary prebiotics, milk fat globule membrane, and lactoferrin affects structural neurodevelopment in the young piglet. *Frontiers in Pediatrics*, 4(February):1–10.

Mudd, A. T., Berding, K., Wang, M., Donovan, S. M., and Dilger, R. N. (2017a). Serum cortisol mediates the relationship between fecal Ruminococcus and brain N-acetylaspartate in the young pig. *Gut Microbes*, 0(0):1–12.

Mudd, A. T. and Dilger, R. N. (2017). Early-life nutrition and neurodevelopment: use of the piglet as a translational model. *Advances in nutrition (Bethesda, Md.)*, 8(1):92–104.

Mudd, A. T., Fil, J. E., Knight, L. C., and Dilger, R. N. (2018b). Dietary Iron Repletion following Early-Life Dietary Iron Deficiency Does Not Correct Regional Volumetric or Diffusion Tensor Changes in the Developing Pig Brain. *Frontiers in Neurology*, 8:735.

Mudd, A. T., Fleming, S. A., Labhart, B., Chichlowski, M., Berg, B. M., Donovan, S. M., and Dilger, R. N. (2017b). Dietary Sialyllactose Influences Sialic Acid Concentrations in the Prefrontal Cortex and Magnetic Resonance Imaging Measures in Corpus Callosum of Young Pigs. *Nutrients*, 9(12):1297.

Mudd, A. T., Getty, C., Sutton, B., and Dilger, R. (2016b). Perinatal choline deficiency delays brain development and alters metabolite concentrations in the young pig. *Nutritional Neuroscience*, 0(0):1–9.

Mudd, A. T., Salcedo, J., Alexander, L. S., Johnson, S. K., Getty, C. M., Chichlowski, M., Berg, B. M., Barile, D., and Dilger, R. N. (2016c). Porcine Milk Oligosaccharides and Sialic Acid Concentrations Vary Throughout Lactation. *Frontiers in Nutrition*, 3(September).

Murrin, L. C., Sanders, J. D., and Bylund, D. B. (2007). Comparison of the maturation of the adrenergic and serotonergic neurotransmitter systems in the brain: Implications for differential drug effects on juveniles and adults. *Biochemical Pharmacology*, 73(8):1225–1236.

Musilova, S., Rada, V., Marounek, M., Nevoral, J., Dušková, D., Bunesova, V., Vlkova, E., and Zelenka, R. (2015). Prebiotic effects of a novel combination of galactooligosaccharides and maltodextrins. *Journal of medicinal food*, 18(6):685–9.

National Research Council (2012). *Nutrient Requirements of Swine: Eleventh Revised Edition*. National Academies Press, Washington, D.C., 11 edition.

Neelima, Sharma, R., Rajput, Y. S., and Mann, B. (2013). Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: A review. *Dairy Science and Technology*, 93(1):21–43.

Nemanic, S., Alvarado, M. C., and Bachevalier, J. (2004). The hippocampal/parahippocampal regions and recognition memory: Insights from visual paired comparison versus object-delayed nonmatching in monkeys. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 24(8):2013–26.

Neufeld, K. M., Kang, N., Bienenstock, J., and Foster, J. A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology and Motility*, 23(3):255–265.

Neuhuber, W. L. (1987). Sensory vagal innervation of the rat esophagus and cardia: a light and electron microscopic anterograde tracing study. *Journal of the Autonomic Nervous System*, 20(3):243–255.

Nicol, C. and Pope, S. (1994). Social learning in sibling pigs. *Applied Animal Behaviour Science*, 40:31–43.

Nielsen, T. R., Kornum, B. R., Moustgaard, A., Gade, A., Lind, N. M., and Knudsen, G. M. (2009). A novel spatial delayed non-match to sample (DNMS) task in the Göttingen minipig. *Behavioural brain research*, 196(1):93–8.

Ninonuevo, M. R., Park, Y., Yin, H., Zhang, J., Ward, R. E., Clowers, B. H., German, J. B., Freeman, S. L., Killeen, K., Grimm, R., and Lebrilla, C. B. (2006). A Strategy for Annotating the Human Milk Glycome.

Nofre, C., Glaser, D., Tinti, J.-M., and Wanner, M. (2002). Gustatory responses of pigs to sixty compounds tasting sweet to humans. *Journal of animal physiology and animal nutrition*, 86:90–96.

Norman, G. and Eacott, M. (2004). Impaired object recognition with increasing levels of feature ambiguity in rats with perirhinal cortex lesions. *Behavioral Brain Research*, 148:79–91.

Nunoya, T., Shibuya, K., Saitoh, T., Yazawa, H., Nakamura, K., Baba, Y., and Hirai, T. (2007). Use of Miniature Pig for Biomedical Research, with Reference to Toxicologic Studies. *Journal of Toxicologic Pathology*, 20(3):125–132.

Nutt, D. and Lawson, C. (1992). Panic Attacks: A neurochemical overview of models and mechanisms. *British Journal of Psychiatry*, 160:165–178.

Oakes, L., Kannass, K., and Shaddy, D. (2002). Developmental changes in endogenous control of attention: The role of target familiarity on infants' distraction latency. *Child Development*, 73:1644–1655.

Odle, J., Lin, X., Jacobi, S. K., Kim, S. W., and Stahl, C. H. (2014). The suckling piglet as an agrimedical model for the study of pediatric nutrition and metabolism. *Annual Review of Animal Biosciences*, 2(1):419–444.

Oliveros, E., Ramirez, M., Vazquez, E., Barranco, A., Gruart, A., Delgado-Garcia, J. M., Buck, R., Rueda, R., and Martin, M. J. (2016). Oral supplementation of 2 -fucosyllactose during lactation improves memory and learning in rats. *The Journal of Nutritional Biochemistry*, 31:20–27.

O'Mahony, S. M., Clarke, G., Borre, Y. E., Dinan, T. G., and Cryan, J. F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behavioural Brain Research*, 277:32–48.

Overduin, J., Schoterman, M. H. C., Calame, W., Schonewille, A. J., and Ten Bruggencate, S. J. M. (2013). Dietary galacto-oligosaccharides and calcium: effects on energy intake, fat-pad weight and satiety-related, gastrointestinal hormones in rats. *British Journal of Nutrition*, 109(07):1338–1348.

Pacioni, G. (1986). Truffle hunting in Italy. *Bulletin of the British Mycological Society*, 20(1):50–51.

Palmano, K., Rowan, A., Guillermo, R., Guan, J., and McJarrow, P. (2015). The Role of Gangliosides in Neurodevelopment. *Nutrients*, 7(5):3891–3913.

Panasevich, M. R., Wankhade, U. D., Chintapalli, S. V., Shankar, K., and Rector, R. S. (2018). Cecal versus fecal microbiota in Ossabaw swine and implications for obesity. *Physiological Genomics*, 50(5):355–368.

Pascalis, O., de Haan, M., Nelson, C., and de Schonen, S. (1998). Long-term recognition memory for faces assessed by visual paired comparison in 3- and 6-month-old infants. *Journal of Experimental Psychology: Learning, Memory, and Cognition*, 24:249–26.

Pascalis, O. and de Schonen, S. (1994). Recognition memory in 3-to-4-day-old human neonates. *NeuroReport*, 5:1721–1724.

Peng, L., He, Z., Chen, W., Holzman, I. R., and Lin, J. (2007). Effects of butyrate on intestinal barrier function in a Caco-2 cell monolayer model of intestinal barrier. *Pediatric research*, 61(1):37–41.

Perry, G. (1992). Olfaction and taste. In Phillips, C. and Piggins, D., editors, *Farm Animals and the Environment*, pages 186–199. C.A.B. International, Wallingford.

Peuranen, S., Tiihonen, K., Apajalahti, J., Kettunen, A., Saarinen, M., and Rautonen, N. (2004). Combination of polydextrose and lactitol affects microbial ecosystem and immune responses in rat gastrointestinal tract. *The British journal of nutrition*, 91(6):905–914.

Pitsikas, N., Rigamonti, A. E., Cella, S. G., and Muller, E. E. (2003). The GABAB receptor and recognition memory: possible modulation of its behavioral effects by the nitrergic system. *Neuroscience*, 118(4):1121–7.

Playne, M. and Crittenden, R. (2009). Galacto-oligosaccharides and other products derived from lactose. In McSweeney, P. and Fox, P. F., editors, *Advanced Dairy Chemistry*, volume 3, chapter 5, pages 121–201. Springer New York, New York, NY.

Popov, N., Toffano, G., Riechert, U., and Matthies, H. (1989). Effects of intraventricularly applied gangliosides and N-acetylneuraminic acid on acquisition and retention performance of a brightness discrimination task in rats. *Pharmacology, Biochemistry and Behavior*, 34(2):209–212.

Probert, H. M., Apajalahti, J. H. A., Rautonen, N., Stowell, J., and Gibson, G. R. (2004). Polydextrose, lactitol, and fructo-oligosaccharide fermentation by colonic bacteria in a three-stage continuous culture system. *Applied and Environmental Microbiology*, 70(8):4505–4511.

Puccio, G., Alliet, P., Cajozzo, C., Janssens, E., Corsello, G., Sprenger, N., Wernimont, S., Egli, D., Gosoniu, L., and Steenhout, P. (2017). Effects of infant formula with human milk oligosaccharides on growth and morbidity: A randomized multicenter trial. *Journal of Pediatric Gastroenterology and Nutrition*, 64(4):624–631.

Purvis, J. M., Clandinin, M. T., and Hacker, R. R. (1982). Fatty acid accretion during perinatal brain growth in the pig. A model for fatty acid accretion in human brain. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 72(2):195–199.

Radke, M., Picaud, J. C., Loui, A., Cambonie, G., Faas, D., Lafeber, H. N., De Groot, N., Pecquet, S. S., Steenhout, P. G., and Hascoet, J. M. (2017). Starter formula enriched in prebiotics and probiotics ensures normal growth of infants and promotes gut health: A randomized clinical trial. *Pediatric Research*, 81(4):622–631.

Radlowski, E. C., Conrad, M. S., Lezmi, S., Dilger, R. N., Sutton, B., Larsen, R., and Johnson, R. W. (2014). A neonatal piglet model for investigating brain and cognitive development in small for gestational age human infants. *PloS one*, 9(3):e91951.

Rho, J. M. and Storey, T. W. (2001). Molecular ontogeny of major neurotransmitter receptor systems in the mammalian central nervous system: norepinephrine, dopamine, serotonin, acetylcholine, and glycine. *J Child Neurol.*, 16(4):271–280.

Richer, J., Lacoste, L., Faure, J., Hauet, T., Ferrie, J., and Carretier, M. (1998). Sacrococcygeal and transsacral epidural anesthesia in the laboratory pig. *Surgical Radiologic anatomy*, 20:431–435.

Rivero-Urgell, M. and Santamaria-Orleans, A. (2001). Oligosaccharides: Application in infant food. *Early Human Development*, 65(SUPPL. 2):43–52.

Röhrig, C. H., Choi, S. S. H., and Baldwin, N. (2017). The nutritional role of free sialic acid, a human milk monosaccharide, and its application as a functional food ingredient. *Critical Reviews in Food Science and Nutrition*, 57(5):1017–1038.

Rose, S., Feldman, J., and Jankowski, J. (2007). Developmental aspects of visual recognition memory in infancy. In Oakes, L. and Bauer, P., editors, *Short- and long-term memory in infancy and early childhood*, pages 153–178. Oxford University Press, New York.

Rose, S. a., Feldman, J. F., and Jankowski, J. J. (2004). Infant visual recognition memory. *Developmental Review*, 24(1):74–100.

Rovee-Collier, C. and Cuevas, K. (2009). The development of infant memory. In Courage, M. and Cowan, N., editors, *The Development of Memory in Infancy and Childhood*, pages 11–41. Psychology Press, Hove.

Rozeske, R. R., Evans, A. K., Frank, M. G., Watkins, L. R., Lowry, C. A., and Maier, S. F. (2011). Uncontrollable, but not controllable, stress sesensitizes 5-HT1A receptors in the dorsal raphe nucleus. *Journal of Neuroscience*, 31(40):14107–14115.

Ruddick, J. P., Evans, A. K., Nutt, D. J., Lightman, S. L., Rook, G. A. W., and Lowry, C. A. (2006). Tryptophan metabolism in the central nervous system: medical implications. *Expert reviews in molecular medicine*, 8(20):1–27.

Rudy, J. (2008). *The neurobiology of learning and memory*. Sinauer Associates, Inc Publishers, Sunderland.

Ruff, H. and Lawson, K. (1990). Development of sustained, focuses attention in young children during free play. *Developmental Psychology*, 26:85–93.

Rytych, J. L., Elmore, M. R. P., Burton, M. D., Conrad, M. S., Donovan, S. M., Dilger, R. N., and Johnson, R. W. (2012). Early life iron deficiency impairs spatial cognition in neonatal piglets. *The Journal of Nutririon Ingestive Behavior and Neurosciences*, pages 1–7.

Sakai, F., Ikeuchi, Y., Urashima, T., Fujihara, M., Ohtsuki, K., and Yanahira, S. (2006). Effects of Feeding Sialyllactose and Galactosylated N-Acetylneuraminic Acid on Swimming Learning Ability and Brain Lipid Composition in Adult Rats. *Journal of Applied Glycoscience*, 53(4):249–254.

Sampson, T. R. and Mazmanian, S. K. (2015). Control of brain development, function, and behavior by the microbiome. *Cell Host and Microbe*, 17(5):565–576.

Sangild, P. T. (2006). Gut responses to enteral nutrition in preterm infants and animals. *Society for Experimental Biology and Medicine*, 231(11):1695–711.

Sauleau, P., Lapouble, E., Val-Laillet, D., and Malbert, C.-H. C.-H. (2009). The pig model in brain imaging and neurosurgery. *Animal : an international journal of animal bioscience*, 3(8):1138–51.

Savignac, H. M., Corona, G., Mills, H., Chen, L., Spencer, J. P. E., Tzortzis, G., and Burnet, P. W. J. (2013). Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-d-aspartate receptor subunits and d-serine. *Neurochemistry International*, 63(8):756–764.

Savignac, H. M., Couch, Y., Stratford, M., Bannerman, D. M., Tzortzis, G., Anthony, D. C., and Burnet, P. W. J. (2016). Prebiotic administration normalizes lipopolysaccharide (LPS)-induced anxiety and cortical 5-HT2A receptor and IL1-β levels in male mice. *Brain, Behavior, and Immunity*, 52:120–131.

Savignac, H. M., Kiely, B., Dinan, T. G., and Cryan, J. F. (2014). Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterology and Motility*, 26(11):1615–1627.

Savignac, H. M., Tramullas, M., Kiely, B., Dinan, T. G., and Cryan, J. F. (2015). Bifidobacteria modulate cognitive processes in an anxious mouse strain. *Behavioural Brain Research*, 287:59–72.

Scalabrin, D. M., Mitmesser, S. H., Welling, G. W., Harris, C. L., Marunycz, J. D., Walker, D. C., Bos, N. A., Tölkkö, S., Salminen, S., and Vanderhoof, J. A. (2012). New prebiotic blend of polydextrose and galacto-oligosaccharides has a bifidogenic effect in young infants. *Journal of Pediatric Gastroenterology and Nutrition*, 54(3):343–352.

Schmidt, K., Cowen, P. J., Harmer, C. J., Tzortzis, G., Errington, S., and Burnet, P. W. J. (2015). Prebiotic intake reduces the waking cortisol response and alters emotional bias in healthy volunteers. *Psychopharmacology*, 232(10):1793–1801.

Scott, H., Rogers, M. F., Scott, H. L., Campbell, C., Warburton, E. C., and Uney, J. B. (2017). Recognition memory-induced gene expression in the perirhinal cortex: A transcriptomic analysis. *Behavioural Brain Research*, 328:1–12.

Selkrig, J., Wong, P., Zhang, X., and Pettersson, S. (2014). Metabolic tinkering by the gut microbiome: Implications for brain development and function. *Gut microbes*, 5(3):369–380.

Siegford, J. M., Rucker, G., and Zanella, A. J. (2008). Effects of pre-weaning exposure to a maze on stress responses in pigs at weaning and on subsequent performance in spatial and fear-related tests. *Applied Animal Behaviour Science*, 110(1-2):189–202.

Sierra, C., Bernal, M. J., Blasco, J., Martínez, R., Dalmau, J., Ortuño, I., Espín, B., Vasallo, M. I., Gil, D., Vidal, M. L., Infante, D., Leis, R., Maldonado, J., Moreno, J. M., and Román, E. (2015). Prebiotic effect during the first year of life in healthy infants fed formula containing GOS as the only prebiotic: a multicentre, randomised, double-blind and placebo-controlled trial. *European journal of nutrition*, 54(1):89–99.

Signoret, S., Baldwin, B., Fraser, S., and Hafez, E. (1975). The behavior of domestic animals. In Hafez, E., editor, *The Behavior of Domestic Animals*, pages 295–329. Tindall and Cox, London.

Silva, R. H., Bellot, R. G., Vital, M. A., and Frussa-Filho, R. (1997). Effects of long-term ganglioside GM1 administration on a new discriminative avoidance test in normal adult mice. *Psychopharmacology*, 129(4):322–8.

Silva, R. H., Bergamo, M., and Frussa-Filho, R. (2000). Effects of neonatal ganglioside GM1 administration on memory in adult and old rats. *Pharmacology and Toxicology*, 87(3):120–125.

Silva, R. H., Felicio, L. F., and Frussa-Filho, R. (1999). Ganglioside GM1 attenuates scopolamine-induced amnesia in rats and mice. *Psychopharmacology*, 141(2):111–117.

Silva, R. H., Felicio, L. F., Nasello, A. G., Vital, M. A., and Frussa-Filho, R. (1996). Effect of ganglioside (GM1) on memory in senescent rats. *Neurobiology of aging*, 17(4):583–586.

Simeoni, U., Berger, B., Junick, J., Blaut, M., Pecquet, S., Rezzonico, E., Grathwohl, D., Sprenger, N., Brüssow, H., Szajewska, H., Bartoli, J. M., Brevaut-Malaty, V., Borszewska-Kornacka, M., Feleszko, W., François, P., Gire, C., Leclaire, M., Maurin, J. M., Schmidt, S., Skórka, A., Squizzaro, C., and Verdot, J. J. (2016). Gut microbiota analysis reveals a marked shift to bifidobacteria by a starter infant formula containing a synbiotic of bovine milk-derived oligosaccharides and Bifidobacterium animalis subsp. lactis CNCM I-3446. *Environmental microbiology*, 18(7):2185–2195.

Skinner, B. (1938). *The Behavior Of Organisms: An experimental analysis*. D. Appleton-Centtury Company, Inc., New York.

Smiricky-Tjardes, M. R., Flickinger, E. A., Grieshop, C. M., Bauer, L. L., Murphy, M. R., and Fahey, G. C. (2003). In vitro fermentation characteristics of selected oligosaccharides by swine fecal microflora. *Journal of animal science*, 81(10):2505–14.

Sommerville, B. and Broom, D. (1998). Olfactory awareness. *Applied Animal Behaviour Science1*, 57:269–286.

Spichtig, V., Michaud, J., and Austin, S. (2010). Determination of sialic acids in milks and milk-based products. *Analytical Biochemistry*, 405(1):28–40.

Stanton, H. and Mersmann, H. (1986). *Swine in cardiovascular research*, volume 36. CRC Press, Boca Raton.

Stanton, M. (1992). Animal models of cognitive development in neurotoxicology. In Isaacson, R. L. and Jensen, K. F., editors, *The Vulnerable Brain and Environmental Risks*, pages 129–149. Springer US, Boston, MA.

Steenbergen, L., Sellaro, R., van Hemert, S., Bosch, J. A., and Colzato, L. S. (2015a). A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. *Brain, Behavior, and Immunity*, 48:258–264.

Steenbergen, L., Sellaro, R., van Hemert, S., Bosch, J. A., and Colzato, L. S. (2015b). A randomized controlled trial to test the effect of multispecies probiotics on cognitive reactivity to sad mood. *Brain, Behavior, and Immunity*, 48:258–264.

Stefanko, D. P., Barrett, R. M., Ly, A. R., Reolon, G. K., and Wood, M. A. (2009). Modulation of long-term memory for object recognition via HDAC inhibition. *Proceedings of the National Academy of Sciences*, 106(23):9447–9452.

Stilling, R. M., van de Wouw, M., Clarke, G., Stanton, C., Dinan, T. G., and Cryan, J. F. (2016). The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? *Neurochemistry International*, 99:110–132.

Sukumar, R., Rose, S. P. R., and Burgoyne, R. D. (1980). Increased Incorporation of [3H]Fucose into Chick Brain Glycoproteins Following Training on a Passive Avoidance Task. *Journal of Neurochemistry*, 34(4):1000–1006.

Sullivan, S., Friess, S. H., Ralston, J., Smith, C., Propert, K. J., Rapp, P. E., and Margulies, S. S. (2013). Improved behavior, motor, and cognition assessments in neonatal piglets. *Journal of neurotrauma*, 30(20):1770–9.

Sun, J., Ling, Z., Wang, F., Chen, W., Li, H., Jin, J., Zhang, H., Pang, M., Yu, J., and Liu, J. (2016a). Clostridium butyricum pretreatment attenuates cerebral ischemia/reperfusion injury in mice via anti-oxidation and anti-apoptosis. *Neuroscience letters*, 613:30–5.

Sun, J., Wang, F., Ling, Z., Yu, X., Chen, W., Li, H., Jin, J., Pang, M., Zhang, H., Yu, J., and Liu, J. (2016b). Clostridium butyricum attenuates cerebral ischemia/reperfusion injury in diabetic mice via modulation of gut microbiota. *Brain research*, 1642:180–188.

Svennerholm, L., Boström, K., Fredman, P., Månsson, J. E., Rosengren, B., and Rynmark, B. M. (1989). Human brain gangliosides: developmental changes from early fetal stage to advanced age. *Biochimica et biophysica acta*, 1005(2):109–17.

Sweasey, D., Patterson, D. S. P., and GLANCY, E. M. (1976). Biphasic myelination and the fatty acid composition of cerebrosides and cholesterol esters in the developing central nervous system of the domestic pig. *Journal of Neurochemistry*, 27(2):375–380.

Talling, J., Waran, N., Wathes, C., and Lines, J. (1998). Sound avoidance by domestic pigs depends upon characteristics of the signal. *Applied Animal Behaviour Science*, 58(3-4):255–266.

Tamis-LeMonda, C. and Bornstein, M. (1989). Habituation and maternal encouragement of attention in infancy as predictors of toddler language, play, and representational competence. *Child Development*, 60:738–751.

Tanida, H. and Nagano, Y. (1998). The ability of miniature pigs to discriminate between a stranger and their familiar handler. *Applied Animal Behaviour Science*, 56:1–4.

Tanida, H., Senda, K.-i., Suzuki, S., Tanaka, T., and Yoshimoto, T. (1991). Color discrimination in weanling pigs. *Animal Science Technology*, 62(11):1029–1034.

Tarr, A. J., Galley, J. D., Fisher, S. E., Chichlowski, M., Berg, B. M., and Bailey, M. T. (2015). The prebiotics 3'Sialyllactose and 6'Sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: Evidence for effects on the gut-brain axis. *Brain, Behavior, and Immunity*, 50:166–177.

Ten Bruggencate, S. J., Bovee-Oudenhoven, I. M., Feitsma, A. L., van Hoffen, E., and Schoterman, M. H. (2014). Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. *Nutrition Reviews*, 72(6):377–389.

Tiihonen, K. K., R yti , H., Putaala, H., and Ouwehand, A. C. (2011). Polydextrose functional fibre Improving digestive health, satiety and beyond. *Nutrafoods*, 10(2):23–28.

Tillisch, K., Labus, J., Kilpatrick, L., Jiang, Z., Stains, J., Ebrat, B., Guyonnet, D., Legrain-Raspaud, S., Trotin, B., Naliboff, B., and Mayer, E. A. (2013). Consumption of fermented milk product with probiotic modulates brain activity. *Gastroenterology*, 144(7):1394–1401.e4.

Urashima, T., Saito, T., Nakamura, T., and Messer, M. (2001). Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate Journal*, 18:357–371.

Van de Weerd, H., Docking, C., Day, J., Avery, P., and Edwards, S. (2003). A systematic approach towards developing environmental enrichment for pigs. *Applied Animal Behavior Science*, 84:101–118.

Van Leeuwen, S. S., Kuipers, B. J. H., Dijkhuizen, L., and Kamerling, J. P. (2016). Comparative structural characterization of 7 commercial galacto-oligosaccharide (GOS) products. *Carbohydrate Research*, 425:48–58.

Vandenplas, Y., Berger, B., Carnielli, V. P., Ksiazyk, J., Lagström, H., Sanchez Luna, M., Migacheva, N., Mosselmans, J.-M., Picaud, J.-C., Possner, M., Singhal, A., and Wabitsch, M. (2018). Human Milk Oligosaccharides: 2'-Fucosyllactose (2'-FL) and Lacto-N-Neotetraose (LNnT) in Infant Formula. *Nutrients*, 10(9).

Vandenplas, Y., De Greef, E., Devreker, T., Veereman-Wauters, G., and Hauser, B. (2013). Probiotics and prebiotics in infants and children. *Current Infectious Disease Reports*, 15(3):251–262.

Vazquez, E., Barranco, A., Ramirez, M., Gruart, A., Delgado-Garcia, J. M., Jimenez, M. L., Buck, R., and Rueda, R. (2016). Dietary 2'-fucosyllactose enhances operant conditioning and long-term potentiation via gut-brain communication through the vagus nerve in rodents. *PLoS ONE*, 11(11):1–14.

Vázquez, E., Barranco, A., Ramírez, M., Gruart, A., Delgado-García, J. M., Martínez-Lara, E., Blanco, S., Martín, M. J., Castanys, E., Buck, R., Prieto, P., and Rueda, R. (2015). Effects of a human milk oligosaccharide, 2 -fucosyllactose, on hippocampal long-term potentiation and learning capabilities in rodents. *Journal of Nutritional Biochemistry*, 26(5):455–465.

Vecsey, C. G., Hawk, J. D., Lattal, K. M., Stein, J. M., Fabian, S. A., Attner, M. A., Cabrera, S. M., Mcdonough, C. B., Brindle, P. K., Abel, T., and Wood, M. A. (2007). Behavioral/Systems/Cognitive Histone Deacetylase Inhibitors Enhance Memory and Synaptic Plasticity via CREB: CBP-Dependent Transcriptional Activation.

Vickers, M. H., Guan, J., Gustavsson, M., Krägeloh, C. U., Breier, B. H., Davison, M., Fong, B., Norris, C., McJarrow, P., and Hodgkinson, S. C. (2009). Supplementation with a mixture of complex lipids derived from milk to growing rats results in improvements in parameters related to growth and cognition. *Nutrition Research*, 29(6):426–435.

Vogt, J. A. and Wolever, T. M. (2003). Fecal acetate is inversely related to acetate absorption from the human rectum and distal colon. *American Society for Nutritional Sciences*, 133(10):3145–3148.

Wainwright, P. E. and Colombo, J. (2006). Nutrition and the development of cognitive functions: interpretation of behavioral studies in animals and human infants. *The American journal of clinical nutrition*, 84(5):961–70.

Wainwright, P. E., Lomanowska, A. M., McCutcheon, D., Park, E. J., Clandinin, M. T., and Ramanujam, K. S. (2007). Postnatal dietary supplementation with either gangliosides or choline: Effects on spatial short-term memory in artificially-reared rats. *Nutritional Neuroscience*, 10(1-2):67–77.

Wako Pure Chemical Industries, L. (2012). HR Series NEFA-HR (2).

Wang, B. (2012). Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. *Advances in nutrition (Bethesda, Md.)*, 3(3):465S–72S.

Wang, B., Hu, H., Yu, B., and Troy, F. A. (2007a). Molecular characterization of pig UDP-N-acetylglucosamine-2-epimerase/N- acetylmannosamine kinase (Gne) gene: Effect of dietary sialic acid supplementation on gene expression in piglets. 5(4):165–175.

Wang, B., Yu, B., Karim, M., Hu, H., Sun, Y., McGreevy, P., Petocz, P., Held, S., and Brand-Miller, J. (2007b). Dietary sialic acid supplementation improves learning and memory in piglets. *The American journal of clinical nutrition*, 85(2):561–9.

Wang, F. B. and Powley, T. L. (2000). Topographic inventories of vagal afferents in gastrointestinal muscle. *The Journal of Comparative Neurology*, 421(3):302–324.

Wang, H. and Peng, R.-Y. (2016). Basic roles of key molecules connected with NMDAR signaling pathway on regulating learning and memory and synaptic plasticity. *Military Medical Research*, 3(1):26.

Wang, M. and Donovan, S. M. (2015). Human microbiota-associated swine: current progress and future opportunities. *ILAR Journal*, 56(1):63–73.

Waworuntu, R., Hain, H., Chang, Q., Thiede, L., Hanania, T., and Berg, B. (2014). Dietary prebiotics improve memory and social interactions while reducing anxiety when provided early in life to normally developing rodents. In *The FASEB Journal*.

Waworuntu, R. V., Hanania, T., Boikess, S. R., Rex, C. S., and Berg, B. M. (2016). Early life diet containing prebiotics and bioactive whey protein fractions increased dendritic spine density of rat hippocampal neurons. *International Journal of Developmental Neuroscience*, 55:28–33.

Wei, T. and Simko, V. (2016). corrplot: Visualization of a correlation matrix. R package version 0.77.

Westbrook, S. R., Brennan, L. E., and Stanton, M. E. (2014). Ontogeny of object versus location recognition in the rat: Acquisition and retention effects. *Developmental psychobiology*, 56(7):1492–506.

Wikoff, W. R., Anfora, A. T., Liu, J., Schultz, P. G., Lesley, S. A., Peters, E. C., and Siuzdak, G. (2009). Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proceedings of the National Academy of Sciences of the United States of America*, 106(10):3698–703.

Willard, J. G., Willard, J. C., Wolfram, S. A., and Baker, J. P. (1977). Effect of diet on cecal pH and feeding behavior of horses. *Journal of Animal Science*, 45(1).

Williams, S., Chen, L., Savignac, H. M., Tzortzis, G., Anthony, D. C., and Burnet, P. W. (2016). Neonatal prebiotic (BGOS) supplementation increases the levels of synaptophysin, GluN2A-subunits and BDNF proteins in the adult rat hippocampus. *Synapse*, 70(3):121–124.

Wood-Gush, D., Vestergaards, K., and Volker Peterson, H. (1990). The significance of motivation and environment in the development of exploration in pigs. *Biology of Behavior*, 15:39–52.

Wood-Gush, D. G. M. and Vestergaard, K. (1991). The seeking of novelty and its relation to play. *Animal Behaviour*, 42(4):599–606.

Xu, X. and Zhu, T. (2005). Effect of ganglioside in repairing the neurological function of chinese children with cerebral palsy. *Chinese Journal of Clinical Rehabiliation*, 9:122–123.

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., Nagler, C. R., Ismagilov, R. F., Mazmanian, S. K., and Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell*, 161(2):264–276.

Yen, C.-H., Wang, C.-H., Wu, W.-T., and Chen, H.-L. (2017). Fructo-oligosaccharide improved brain β-amyloid, β-secretase, cognitive function, and plasma antioxidant levels in D-galactose-treated Balb/cJ mice. *Nutritional Neuroscience*, 20(4):228–237.

Yen, J. (2001). Anatomy of the Digestive System and Nutritional Physiology. In Lewis, A. and Southern, L. L., editors, *Biology of the Domestic Pig*, chapter 3, pages 31–63. Cornell University Press, Ithaca, second edi edition.

Zatz, M. and Barondes, S. H. (1971). Rapid Transport of Fucosyl Glycoproteins To Nerve Endings in Mouse Brain. *Journal of Neurochemistry*, 18(6):1125–1133.

Zeisel, S. H. (2004). Nutritional Importance of Choline for Brain Development. *Journal of the American College of Nutrition*, 23(sup6):621S–626S.

Zeng, H., Grapov, D., Jackson, M. I., Fahrmann, J., Fiehn, O., Combs, G. F., and Jr (2015). Integrating Multiple Analytical Datasets to Compare Metabolite Profiles of Mouse Colonic-Cecal Contents and Feces. *Metabolites*, 5(3):489–501.

Zonderland, J. J., Cornelissen, L., Wolthuis-Fillerup, M., and a.M. Spoolder, H. (2008). Visual acuity of pigs at different light intensities. *Applied Animal Behaviour Science*, 111(1-2):28–37.